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THE EFFECT OF PYGEUM AFRICANUM ON FIBROBLAST GROWTH FACTOR (FGF) AND TRANSFORMING GROWTH FACTOR BETA (TGF β 1/LAP) EXPRESSION IN ANIMAL MODEL

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On the basis of its fibroblast growth factor (FGF) inhibitory effect we assessed the possible inhibitory anti-inflammatory role of Pygeum Africanum extract (Tadenan) on FGF and transforming growth factor beta (TGF β 1/LAP) expression of macrophages and neutrophils in broncho-alveolar lavage fluid (BAL) of rats in a bleomycin-induced acute inflammation model. The rats were divided into three groups: 17 untreated controls, 10 bleomycin-instilled rats, receiving NaCl (0.9%), and 10 rats receiving Pygeum Africanum extract. On the 12th (and 15th day) we performed BAL and after labelling of cells expression of FGF and TGF β 1 (LAP) was measured by flow-cytometry. We made a quantitative analysis of BAL cells as well. One-way ANOVA was used for statistical analysis. We found in Pygeum Africanum extract treated group 1, a significantly decreased number of neutrophil granulocytes ($p < 0.05$) compared with other groups 2, there was a considerable decrease (not significant) in expression of TGF β 1(LAP) on BAL macrophages, but not in case of FGF. In conclusion: our results show the possible 1. inhibitory effect of this drug on TGF β 1 (LAP) expression, 2. anti-inflammatory role on neutrophil granulocytes.

Keywords: fibroblast growth factor, transforming growth factor beta, anti-inflammatory drug

Lung fibrosis induced by an intratracheal instillation of bleomycin in laboratory rodents is attributed to proliferation of lung fibroblasts. It is admitted that alveolar macrophages are the culprits of this overactivity by producing TNF α , TGF β 1 and FGF.

The extract of *Pygeum Africanum* has a primary inhibitory effect on FGF. It is used in clinical practice in the therapy of benign prostatic hyperplasia [1, 2].

In the present work we assessed the possible inhibitory, anti-inflammatory effect of *Pygeum Africanum* extract on FGF and LAP expression of macrophages and neutrophil granulocytes in bronchoalveolar lavage fluid (BAL) of rats in a bleomycin-induced acute inflammation model [2, 3].

Materials and methods

Animals

Adults male rats of Wistar strain (IFFA CREDOFr.), weighing 246 ± 56 g at the start of the experiment were maintained in air filtered and ventilated boxes.

They were distributed into three groups: 17 untreated controls (statistically optimised number), bleomycin-instilled rats receiving 3 days later either Tadenan (10 rats) or saline administration (10 rats).

Number of controls = Number of rats in each treated group $\times$ square root of the number of treated groups.

Anaesthesia

Rats were food-starved during one day before the experiment; water was given *ad libitum* during starvation. They were anaesthetized by intraperitoneal injection of sodium pentobarbital (38 mg/kg body weight).

Bleomycin administration

Bleomycin (Bleomycin sulphate USA 15 mg/vial) was dissolved in 8 ml sterile saline. Bleomycin was given at the dose of 1.2 mg/m^2 for each animal [3]. Rats were anaesthetized, kept on a warming blanket. After hair shaving and skin cleaning on the neck, the trachea was denuded and bleomycin was instilled transtracheally between two cartilage rings; so an exact dose of bleomycin was inhaled by the rats (first day of the experiment).

*Administration of *Pygeum Africanum* extract or saline*

On the third day of experiment, sterile saline or *Pygeum Africanum* extract (Tadenan á 50 mg, Lab. Fournier) were administered orally by the way of a Carriertube to awake rats. We calculated the dose of *Pygeum* for rats in $1.43 \text{ mg/body weight}$

(on the basis of the daily dose given to humans/70 kg body weight), and pygeum extract was diluted in paraffin oil. Saline (0.9%) was given by the same way, and the same dose.

Broncho-alveolar lavage (BAL)

On the twelfth/fifteenth day of experiment, thorax was opened and the lungs were carefully excised and placed flat on a Petri dish to avoid gravity differences between the different lobes. During excision, a Carrieri cannula was introduced into the trachea. Lungs were then washed through the cannula with 30 ml of warmed (37 °C) sterile saline. Six 5 ml syringes were used successively, each being pushed and pulled 5 times, during 15 minutes. A push-pull lasted 30 seconds. The liquids of lavage were pooled and centrifuged at 250 g for 15 min. at 4 °C. The pellets were then resuspended in 1 ml of phosphate buffer solution (Dulbecco's PBS without Ca, Mg, Na. SigmaD5652) for a count of neutrophil granulocytes using the Thoma method.

Staining

Trypan blue was used to detect living cells and May-Grunwald for identifying neutrophils and macrophages in BAL.

To make a differential count of BAL cells we used cytopsin method (200,000 cell/ml) with 200 µl of cell suspension at 170 g (900 rpm) for 15 minutes. BAL smears were stained by Papanicolau method for counting.

Labelling techniques

We used

1. Mouse anti-human FGF-basic Sigma 0.2 ml F 6162 monoclonal antibody.
2. Mouse anti-human LAP (TGFβ1) monoclonal neutralizing antibody (Rλ D 500 µg MAB 246).
3. Isotype control - IgG1 anti-mouse DAKO A/S XD931 Denmark.

Method of labelling

It consisted of two successive labellings. First, one antibody was added to the sample at the dose of 1 µg/million of suspended cells for one hour. Then the suspension was washed twice with PBS. A second FITC labelled antibody was added at the dose of 10 µl/100 µl of the cell suspension for half an hour. It was washed twice and centrifuged at 400 g for 5 minutes.

Flow-cytometry

It was performed on cell suspensions containing $0.2 \times 10^6/\text{ml}$ by a cell sorter (OrthoCyte by OrthoDiagnostic Systems RoissyFr). 100 μl of FITC labelled antibody-cell suspension was analysed at 520 nm and 560 nm using an excitation wavelength of 488 nm. Cells were identified according to the cell size and on the basis of nucleus/cytoplasm ratio. Cell subpopulations of BAL were screened on a two axis graph.

Statistics

We used one-way ANOVA to evaluate the differences between the three animal groups according to proportion of cells in BAL.

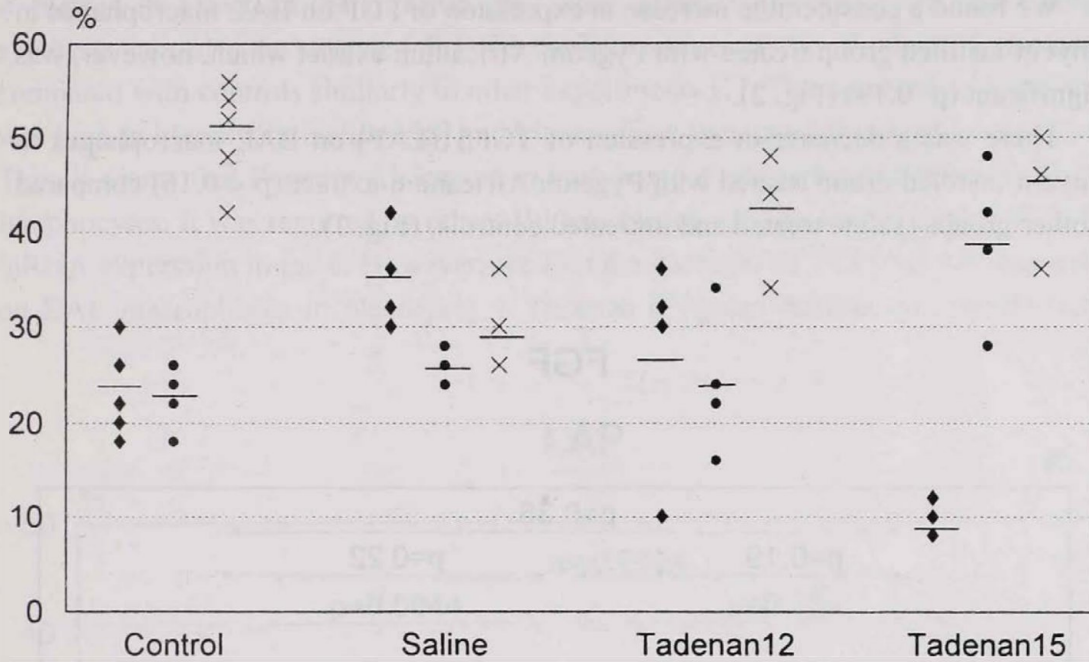
Table I

Number of BAL cells

	Neutrophil	Lymphocyte	Monocyte
Control	$4 \times 10^4/\text{ml}$	$4.8 \times 10^4/\text{ml}$	$8.4 \times 10^4/\text{ml}$
	$5.2 \times 10^4/\text{ml}$	$3.6 \times 10^4/\text{ml}$	$11.2 \times 10^4/\text{ml}$
	$5.2 \times 10^4/\text{ml}$	$4.4 \times 10^4/\text{ml}$	$10.4 \times 10^4/\text{ml}$
	$4.4 \times 10^4/\text{ml}$	$4.8 \times 10^4/\text{ml}$	$10.8 \times 10^4/\text{ml}$
	$3.6 \times 10^4/\text{ml}$	$4.4 \times 10^4/\text{ml}$	$11.2 \times 10^4/\text{ml}$
Saline	$6 \times 10^4/\text{ml}$		$9.6 \times 10^4/\text{ml}$
	$6 \times 10^4/\text{ml}$	$4.8 \times 10^4/\text{ml}$	$5.2 \times 10^4/\text{ml}$
	$6.4 \times 10^4/\text{ml}$	$5.2 \times 10^4/\text{ml}$	$6.0 \times 10^4/\text{ml}$
	$7.2 \times 10^4/\text{ml}$	$5.6 \times 10^4/\text{ml}$	$7.2 \times 10^4/\text{ml}$
	$8.4 \times 10^4/\text{ml}$	$4.8 \times 10^4/\text{ml}$	$5.2 \times 10^4/\text{ml}$
Tadenan			
12. day	$6.4 \times 10^4/\text{ml}$	$3.2 \times 10^4/\text{ml}$	$8.8 \times 10^4/\text{ml}$
	$6.0 \times 10^4/\text{ml}$	$4.4 \times 10^4/\text{ml}$	$8.8 \times 10^4/\text{ml}$
	$7.2 \times 10^4/\text{ml}$	$4.8 \times 10^4/\text{ml}$	$6.8 \times 10^4/\text{ml}$
15. day	$1.6 \times 10^4/\text{ml}$	$7.6 \times 10^4/\text{ml}$	$10.0 \times 10^4/\text{ml}$
	$1.6 \times 10^4/\text{ml}$	$5.6 \times 10^4/\text{ml}$	$10.0 \times 10^4/\text{ml}$
	$2.4 \times 10^4/\text{ml}$	$8.4 \times 10^4/\text{ml}$	$9.2 \times 10^4/\text{ml}$
	$2.1 \times 10^4/\text{ml}$	$6.8 \times 10^4/\text{ml}$	$9.6 \times 10^4/\text{ml}$
	$2.0 \times 10^4/\text{ml}$	$9.6 \times 10^4/\text{ml}$	$7.2 \times 10^4/\text{ml}$

Correlation analysis

Values were tested for correlation in each group. Groups of bleomycin-instilled (Pygeum Africanum extract or saline treated) rats and controls were analysed by one-way ANOVA (two sample *t*-test).



	C-T12	C-T15	S-T12	S-T15	T12-T15
◆ Neutrophil	p=0.3080	p=0.0001	p=0.1375	p=0.0005	p=0.0270
● Lymphocyte	p=0.3760	p=0.0131	p=0.3604	p=0.0227	p=0.0189
✕ Monocyte	p=0.0273	p=0.0983	p=0.0077	p=0.0983	p=0.2629

Fig. 1. Distribution of BAL cells

Results

On the basis of earlier data on the twelfth day and a few days later (15th day) we performed BAL in each animal group. First we made a quantitative analysis of BAL cells. We found significantly decreased number of neutrophil granulocytes ($p < 0.05$) in bleomycin-instilled group treated with Pygeum Africanum extract (Tadenan) compared

with other groups (saline treated and untreated control) on the twelfth/fifteenth day. According to lymphocytes, we found a significant increase in number on twelfth/fifteenth day in Tadenan treated group, but a not considerable decrease in number of monocytes compared with saline treated group (Table I, Fig. 1).

After labelling of cells we measured expression of FGF and LAP by flow-cytometry.

We found a considerable increase in expression of FGF on BAL macrophages in bleomycin-instilled group treated with *Pygeum Africanum* extract which, however, was not significant ($p=0.19$) (Fig. 2).

There was a decrease in expression of TGF β 1(LAP) on BAL macrophages in bleomycin-instilled group treated with *Pygeum Africanum* extract ($p < 0.16$) compared with other groups (saline treated and untreated controls) (Fig. 3).

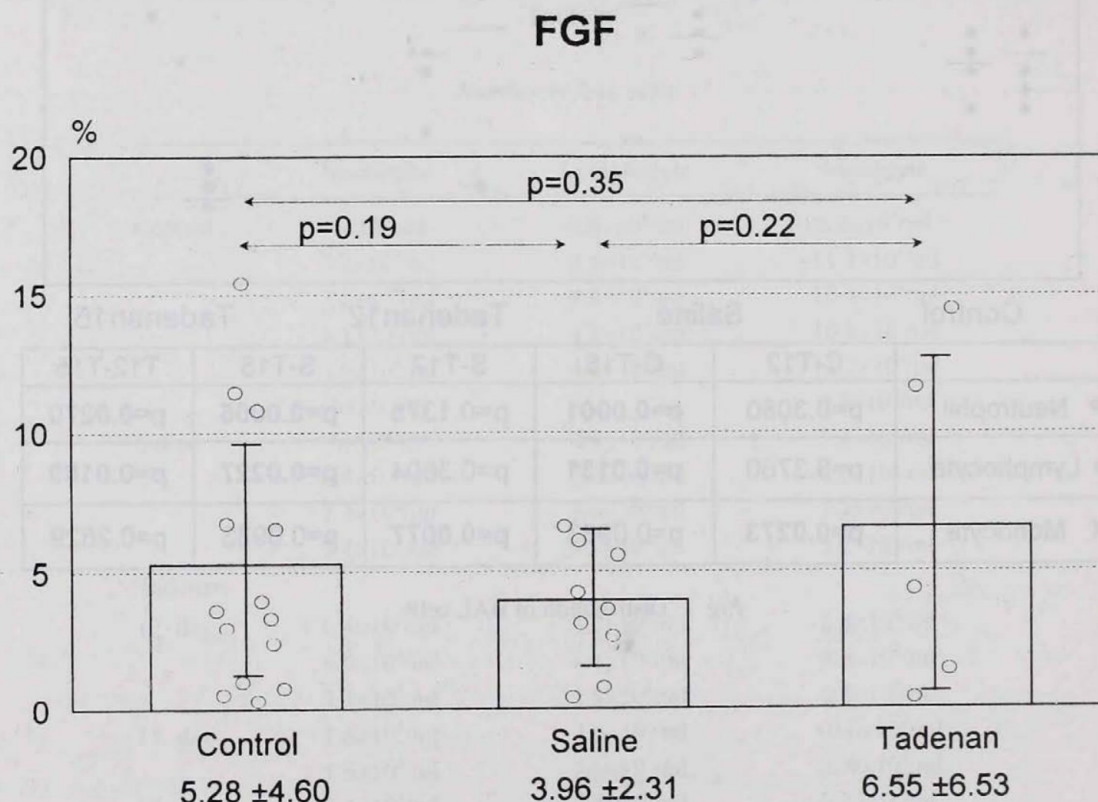


Fig. 2. FGF expression on BAL cells

Discussion

On the basis of bleomycin model established by Thrall et al. [3, 4] and others [5] we could induce acute inflammation in rat lung. It is known that bleomycin induces granulocytosis [6], thus decreasing effect on number of neutrophil granulocytes could be attributed to *Pygeum Africanum* extract. The mechanism is unknown. In this model, it exerted its effect on the fifteenth day, when inflammation is severe, and collagen synthesis can also be detected [6]. Bleomycin increased the number of monocytes compared with controls similarly to other experiments [7]. The number of lymphocytes was high in bleomycin-instilled [8] and bleomycin + *Pygeum Africanum* treated group. Thus it seems that *Pygeum Africanum* extract has not any antiinflammatory effect on lymphocytes. It was reported by others [9] that bleomycin can induce higher TGF beta mRNA expression in mice. However, we found a decrease of TGF β 1(LAP) expression on BAL macrophages in bleomycin + Tadenan (*Pygeum Africanum* extract) treated

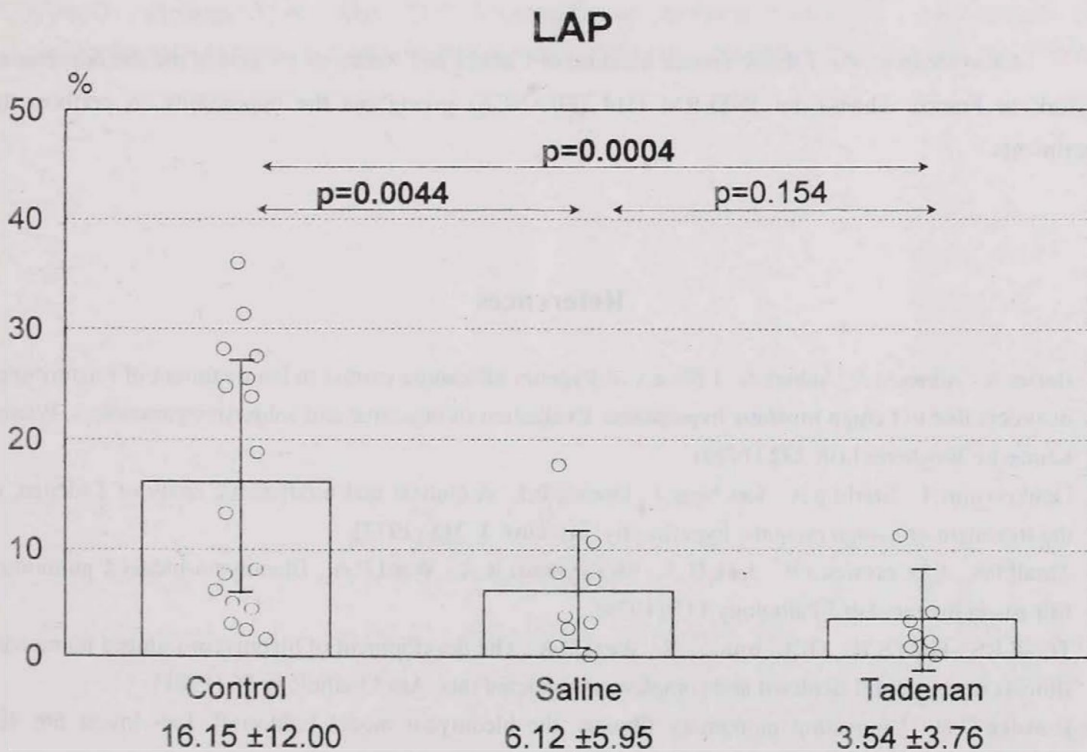


Fig. 3. TGF β 1 (LAP) expression on BAL cells

group, which may underline its anti-inflammatory effect. We found no correlation between Tadenan (Pygeum Africanum extract) and bleomycin induced effect on lymphocytes. It was reported by others [9] that bleomycin can induce higher TGF beta mRNA expression in mice. However, we found a decrease of TGF β 1(LAP) expression on BAL macrophages in bleomycin + Tadenan (Pygeum Africanum extract) treated group, which may underline its anti-inflammatory effect. We found no correlation between Tadenan (Pygeum Africanum extract) and bleomycin induced increased FGF expression. Thus, it did not have inhibitory effect on FGF in this model, as it did in others [1]. This is the first animal model study concerning the anti-inflammatory effect of Pygeum Africanum extract on lung BAL cells. Taken together, theoretically Pygeum Africanum extract could be an alternative antifibrotic drug other compounds [6, 10–15].

In summary: our results show the possible anti-inflammatory effect of Pygeum Africanum extract (Tadenan) on neutrophil granulocytes in acute inflammation induced by bleomycin. It seems that Pygeum Africanum extract has an inhibitory effect on TGF β 1(LAP) expression of BAL macrophages, but not on FGF.

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The first of these is the fact that the earth is not a perfect sphere, but is flattened at the poles and bulged out at the equator. This is due to the centrifugal force of rotation, which tends to pull the material of the earth away from the poles and towards the equator. The second is the fact that the earth is not a uniform body, but is composed of different layers of material. The third is the fact that the earth is not a rigid body, but is capable of deformation. These three factors are the main causes of the irregularities of the earth's surface.

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FLEROXACIN UPTAKE IN ISCHAEMIC LIMB TISSUE

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Antibiotic application to patients with ischaemia of lower limbs may be indicated to avoid or treat infection of soft tissues. Fleroxacin, a fluoroquinolone, active against various Gram-negative and Gram-positive organisms may be used for this purpose. We evaluated the diffusion of fleroxacin into bone, subcutaneous fat, muscle and tendon tissues of lower limb tissue after a 400 mg i.v. dose. Concentrations in ischaemic tissues were similar to those found in non-ischaemic sites. Since the maximum antibiotic levels found were lower than the MICs of various pathogens relevant for infection, we suggest to increase the dose used for this peri-operative prophylaxis to 800 mg.

Keywords: ischaemic tissues, antibiotic therapy, fleroxacin

Patients suffering of ischaemia of their lower limbs due to arterial occlusive disease often can develop soft tissue infection that may lead to the need for ablation surgery [1]. Surgical intervention itself may be complicated by bacterial infections. *Enterobacteriaceae*, non-fermentative Gram-negative rods, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes* and anaerobic bacteria are among the most common pathogens responsible for such infectious complications [2]. Drugs for antibacterial treatment and prophylaxis should, therefore, have an adequate spectrum and reach effective drug concentrations both in ischaemic and non-ischaemic tissues.

Fleroxacin, a member of the class of fluoroquinolones, has a favourable long-half life time of 9–12 h, favouring once daily administration and is characterized by good interstitial penetration, as measured by diffusion into skin-blister fluid. Furthermore, it has good *in vitro* activity against many Gram-negative and Gram-

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positive organisms [3]. Spectrum of antibacterial *in vitro* activity, side-effects, length of time of adequate concentrations reached at the site of infection are properties of the drug influencing the success of antibacterial treatment. Human and animal studies have suggested that fleroxacin penetrates into most tissues [4–5], but limited information is available on *in vivo* penetration into pathological tissues in humans. To our knowledge, its ability to diffuse into tissues of ischaemic limbs has not been studied so far. It was the aim of this paper to elucidate the ability of fleroxacin to diffuse into bone, subcutaneous fat, muscle and tendon of limb tissues affected by arterial occlusive disease.

Patients and methods

Patients

Thirteen patients (7 males and 6 females), aged 39 to 88 years (mean 67), scheduled for metatarsal amputation due to arterial occlusive disease, were enrolled after giving informed consent.

Drug

All patients received a single i.v. dose of 400 mg fleroxacin. Infusion (over 20 min) was started 2 h before the onset of surgical procedure.

Samples

Approx. 2 h after start of i.v. fleroxacin application, 100–200 mg healthy tissue proximal from the amputation line, 100–200 mg ischaemic tissue distal from the amputation line, and 5 ml of venous blood were collected during the surgical intervention. The blood was clotted and centrifuged to obtain serum. All samples were kept in brown glassware and aluminum foil to protect them from light.

Tissue specimens consisted of muscle, subcutaneous fat, tendon and bone. Na/K phosphate buffer pH 7.2 was added (1:6) to each tissue aliquot which was homogenized using Ultraturax (Ika-Werk Staufen, Staufen, Germany). This suspension was then centrifuged for 15 min at $1,000 \times g$ and the supernatant was removed and frozen at -80°C until assayed. No corrections were made for blood contamination [4].

Assay method

Fleroxacin concentrations in serum and tissue samples were assayed using Antibiotic Medium 1 agar (Difco) plates seeded with *E. coli* Kp 05924. Previously this biological method has been shown to correlate well with an HPLC method [5]. The detection limit of the assay was 0.8 mg fleroxacin/L.

Statistical analysis

The coefficient of variation of the assay was 7.6%. Student's *t*-test for grouped data was used to determine the statistical significance of the differences between mean concentration values. A *p* value of <0.05 was considered to be statistically significant.

Results

Mean and standard deviation of fleroxacin concentrations detected in serum, ischaemic tissues and non-ischaemic tissues are listed in Table I. Table II shows the ratio site/serum of the drug in various tissues. Mean concentrations detected in non-ischaemic tissues were not significantly different from those found in distal ischaemic tissues.

Drug concentrations in non-ischaemic muscle was similar to those in serum, exhibiting a wider range, the others were lower than in serum. Bone specimens yielded the lowest concentrations.

Table I

*Concentrations of fleroxacin in serum and non-ischaemic
and ischaemic limb tissues*

	Non-ischaemic	Ischaemic
Serum	3.99(1.1)	
Fat	2.17(1.40)	2.36(1.20)
Bone	1.04(0.47)	1.15(0.74)
Muscle	4.40(2.16)	2.28(0.93)
Tendon	2.44(1.30)	2.72(0.93)

Values (mg/L or Kg) are expressed as mean and standard deviation

Table II

*Penetration of fleroxacin into non-ischaemic and ischaemic tissues,
expressed as tissue/serum ratio*

	Non-ischaemic tissue	Ischaemic tissue
Fat	0.54	0.59
Bone	0.26	0.28
Muscle	1.10	0.57
Tendon	0.60	0.68

Discussion and conclusions

Our results indicate that approximately 2 hours after i.v. fleroxacin administration tissue concentrations are nearly identical in ischaemic and non-ischaemic tissues. This sampling time was chosen because previously it has been demonstrated that fleroxacin concentrations reach a steady-state in skin-blister fluid after 2 hours and, thereafter remain relatively constant up to 6 hours [6]. The penetration is not significantly affected by ischemia. The diffusion of fleroxacin into tissue was found to be almost independent from the arterial blood flow. This could be explained by the possibility of drug diffusion into ischaemic tissue from venous and lymphatic circulation. These two circulations are not compromised in atherosclerosis; the drug may arrive at capillaries from venous vessels or may diffuse from lymphatic vessels, where the gradient of concentration rules the diffusion. Our findings correlate well with results from other authors who showed that there was no correlation between blood flow and cephadrine or metronidazole diffusion into ischaemic tissues [1]. The low concentration found in the bone tissue could be due by the presence of a blood/bone barrier that hinders the penetration of fleroxacin from the blood to the protein matrix of the bone tissue. In ischaemic muscle, subcutaneous fat and tendon, the concentration levels reached by fleroxacin are about 54–68% of those present in serum, suggesting an unsatisfactory diffusion. Ratio site/serum (%) in bone range among fluoroquinolones from 103% in case of lomefloxacin to 61% for ofloxacin, 44% pefloxacin or 28% for ciprofloxacin; in muscle from 70 to 50% for ciprofloxacin [9]. The low diffusibility of fleroxacin into tissues could be explained by its low liposolubility due to an octanol/phosphate buffer partition of 0.25 at pH 7.4 [10].

The concentrations achieved do not cover all major pathogens responsible for postoperative wound infection of ischaemic limbs. Susceptibility breakpoints of fleroxacin for *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterobacteriaceae* and *Pseudomonas aeruginosa* are less than or equal to 2 mg/L [11]. It seems, therefore, indicated to increase the dosage used for preoperative prophylaxis of infectious complications in ischaemic limbs from 400 to 800 mg.

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QSAR STUDIES ON ANTIMALARIAL 2,4-DIAMINO-6-QUINAZOLINE SULFONAMIDES

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The present paper discusses QSAR studies on antimalarial 2,4-diamino-6-quinazoline sulfonamide derivatives using electronic parameters, namely energy of highest occupied molecular orbitals (E_H), energy of lowest unoccupied molecular orbitals (E_L) and charge density (CD). The results have shown that better results are obtained by introducing dummy parameters (indicator parameter), Ip. Excellent results are obtained when all the four parameters (E_H , E_L , CD and Ip) are used in correlation analysis.

Keywords: QSAR studies, antimalarial activity of 2,4-diamino-6-quinazoline sulfonamides

Elslager and coworkers [1, 2] have synthesized and estimated antimalarial activity of a series of 2,4-diamino-6-quinazoline sulfonamide as shown in Fig. 1.

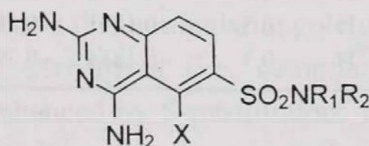


Fig. 1

Substantial antimalarial activity was demonstrated by some of these compounds (see Table I). Compounds were arbitrarily considered to be “active” when they produce

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at least a 100% increase in the mean survival time and the activity is expressed as OMST, which is the mean survival time (days) of control mice. These OMST values, which are also adopted in the present study, are given in Table I.

At this stage it is worth mentioning that extensive work has been reported on antimalarial drugs and their pharmacology, therapeutic efficacy, mode of action and mechanism, clinical pharmacokinetics etc. [1–30].

Table I

2,4-Diamino-6-quinazoline sulfonamides used in the present study, their antimalarial activity (Δ MST) and E_H , E_L , E_T , and CD values at S-atom as well as values of indicator parameter (I_p), ref. Fig. 1

S.N.	NR ₁ R ₂	X	Δ MST	E_H	E_L	E_T	CD	I_p
1	NH ₂	H	0.1	0.6236	-0.1976	31.7784	0.200	0
2	N(CH ₃) ₂	H	8.7	-0.1852	-0.3529	33.4090	0.210	0
3	N(C ₂ H ₅) ₂	H	3.3	0.3401	-0.1954	36.5052	0.196	0
4	N(C ₂ H ₅) ₂	Cl	2.3	0.3391	-0.2220	39.6441	0.155	1
5	N(CH ₂ CH ₂ OH) ₂	H	0.3	0.2123	-0.1975	45.0892	0.187	0
6	N(CH ₃)CH(CH ₃) ₂	H	0.7	-0.1830	-0.4745	42.6764	-0.242	0
7	N(CH ₃)CH ₂ CH ₂ N(C ₂ H ₅) ₂	H	0.1	-0.1830	-0.4745	42.6765	-0.242	0
8	N(CH ₂) ₅	H	4.4	-0.1841	-0.5957	38.4490	-0.245	0
9	[N(CH ₂) ₅]-N(CH ₂) ₅	H	0.3	0.0181	-0.2004	46.7435	0.195	0
10	N[(CH ₂) ₂] ₂ O	H	4.7	0.2708	-0.1964	40.7980	0.920	0
11	N[(CH ₂) ₂] ₂ S	H	2.5	0.1905	-0.1973	38.9040	0.187	0
12	N[(CH ₂) ₂] ₂ NCH ₃	H	1.0	0.1287	-0.1982	41.7166	0.185	0
13	N[(CH ₂) ₂] ₂ NC(=O)OC ₂ H ₅	H	0.2	-0.1847	-0.1942	49.5462	0.185	0
14	2-(C ₆ H ₅ CH ₂)-N(CH ₂) ₅	H	0.3	-0.1960	-0.1454	52.1522	-0.225	0
15	4Cl-C ₆ H ₄ NH	H	0.7	0.3481	-0.1978	46.2185	0.194	1
16	3,4-Cl ₂ -C ₆ H ₃ NH	H	3.1	-0.1855	-0.6402	47.6971	-0.247	1
17	3-Br-C ₆ H ₄ NH	H	0.3	0.5016	-0.1967	44.6294	0.201	1
18	3-Cl-C ₆ H ₄ NCH ₃	H	0.3	-0.1841	-0.3865	45.2710	-0.699	1
19	C ₆ H ₅ NCH ₃	H	0.5	-0.1841	-0.3864	42.1728	-0.699	0
20	3,4-Cl ₂ -C ₆ H ₄ CH ₂ NCH ₃	H	0.3	-0.1854	-0.2962	50.6392	-0.703	1

Excellent reviews on antimalarial drugs also appeared in the literature [26, 31]. However, till date no quantitative structure-activity relationship (QSAR) study on antimalarial 2,4-diamino-6-quinazoline sulfonamide was investigated. In view of this the present study deals with quantitative structure-activity relationship studies on these compounds. The electronic parameters, namely energy of highest occupied molecular orbitals (E_H), energy of lowest unoccupied molecular orbitals (E_L), total π -electron

energy (E_T) and charge density (CD) at sulfur atom of the side chain ($-\text{SO}_2\text{NR}_1\text{R}_2$) were used for the present study. In addition, use is also made of a dummy parameter (indicator parameter, Ip). The results we discussed below.

Materials and methods

The aforementioned parameters, E_H , E_L , E_T and CD, for the series of compounds listed in Table I were calculated using HMO method and are recorded in Table I. QSARs were established by means of stepwise multiple regression analysis using MSTAT software. The biological activities expressed as ΔMST were adopted from the earlier work.

At this stage it is worth mentioning that the aforementioned parameters E_H , E_L , E_T and CD were calculated using Huckel Molecular Orbital (HMO) method [32–34] and that this method is a very primitive one. However, the method is extensively and successfully used in chemistry [35], biochemistry [11, 16] and drug research [36]. It has been observed that for a comparative study on π -electron systems, HMO method is as good as any refined methods [10, 14, 23, 24, 33, 37].

In the present study we have calculated E_H , E_L , E_T and CD using the program HMO 1.1 supplied by Allan Wissner, Wyeth-Ayerst Research, 401 N. Middletown RD, Pearl River, NY. 10965-1215, and the results are reported in β units.

Results and discussion

The 2,4-diamino-6-quinazoline sulfonamide derivatives used in the present study are given in Table I. Antimalarial activity (ΔMST) as well as E_H , E_L , E_T and CD are also given in Table I.

A perusal of Table I shows that antimalarial potency is optimal among lower N-monoalkyl and N₁N-dialkyl derivatives (i.e. compounds 2, 3, 10) and that the antimalarial potency is not enhanced by 5-substitution. The most potent compound is found to be the morpholino analogue 10. The unsubstituted sulfonamide (compound 1) is devoid of antimalarial activity. On the basis of ΔMST the following order of antimalarial activity can be proposed for the compounds under the present study:

$$2 > 10 > 8 > 3 > 16 > 11 > 4 > 12 > 6 = 15 > 19 > 5 = 9 = 14 = 17 = 18 = 20 > 13 > 7 = 1$$

The perusal of Table I also shows that no correlation exists between antimalarial activity and substituent effect at X as well as at R_1R_2 of the side chain fragment - NR_1R_2 .

The perusal of Table I further shows that degeneracy exists in the antimalarial activity as well as the molecular descriptors used. Table II (correlation matrix) shows that there is no correlation between antimalarial activity and any of the individual correlation parameters used (E_H , E_L , E_T and CD). Hence, no univariate regressions are possible for modeling antimalarial activity of the compounds under the present study.

Table II

Correlation matrix for the correlation of antimalarial activity (ΔMST) of a series of 2,4-diamino-6-quinazoline sulfonamides with E_H , E_L , E_T , CD, Ip

	ΔMST	E_H	E_L	E_T	CD	Ip
ΔMST	1.0000					
E_H	0.0158	1.0000				
E_L	-0.0191	0.6640	1.00000			
E_T	-0.5373	-0.3561	-0.1623	1.0000		
CD	0.2393	0.6019	0.6162	-0.3620	1.0000	
Ip	-0.3146	-0.2001	-0.2937	0.6042	-0.5767	1.0000

In view of the aforementioned findings we have subjected the data to multiple regression analysis. Several multiple regressions have indicated that the multiple correlations are improved compared to univariate correlations. However, these improved results too are not statistically. But when we have introduced a dummy parameter (indicator parameter) Ip, good results were obtained. Furthermore, in a multiple regression, wherein four parameters; E_H , E_L , E_T , CD and Ip are involved, the result found to be excellent.

It should be mentioned here that the dummy parameter (Ip) used by us relates to substitution at X and R_1R_2 . If any of these substituents X, R_1R_2 contains halogen then Ip is taken as unity otherwise it (Ip) is zero.

Statistically allowed multiple regression analysis data are presented in Table III. These data show that the terivariate correlation involving E_H , E_L , E_T and CD gives better results. The regression expression for the correlation is found to be as follows

$$\text{Antimalarial activity} = -(5.6236)E_H - (0.2611)E_T + (3.1080)CD + 13.2U42 \quad (1)$$

That means that charge density (CD) plays the dominant role in modeling antimalarial activity using Eq. 1.

When the indicator parameter (Ip) is added to the above regression Eq. 1, the correlation coefficient is dramatically improved from 0.8122 to 0.9019. Out of all the multiple correlations we tried this correlation using E_H , E_L , E_T , CD and Ip is found to be the excellent one. The corresponding regression expression is found to be as follows:

$$\text{Antimalarial activity} = -(8.0229)E_H - (0.3370)E_T + (4.5510)CD + (2.3189)Ip + 15.9012 \quad (2)$$

Table III

Regression parameters and quality of correlations of the antimalarial activity (ΔMST) of 2,4-diamino-6-quinazoline sulfonamide derivatives with various parameters used in the present study

S.N.	Parameters used	A_i $i=1, 2, 3, 4$	B	Standard deviation (SD)	Correlation coefficient (R)	F ratio
1	E_T and IR_1	$A_1 = -0.2407$ $A_2 = 0.2617$	11.9015	1.8735	0.6036	0.487
2	E_H E_T	$A_1 = -2.9770$ $A_2 = -0.2873$	14.1355	1.6967	0.6918	7.804
3	E_H E_T and IR_1	$A_1 = -3.4007$ $A_2 = -0.3192$ $A_3 = 0.8394$	15.2682	1.7060	0.7099	5.418
4	E_H E_T Cd	$A_1 = -5.6236$ $A_2 = -0.2611$ $A_3 = 3.1080$	13.2042	1.4131	0.8122	10.335
5	E_L E_T Cd	$A_1 = -8.1027$ $A_2 = 0.1905$ $A_3 = 2.7530$	7.3536	1.6013	0.7503	6.869
6	E_H E_T Cd and IR_1	$A_1 = -8.0229$ $A_2 = -0.3370$ $A_3 = 4.5510$ $A_4 = 2.3189$	15.9012	1.0805	0.9019	16.351

The importance of indicator parameter (Ip) is further demonstrated by the data presented in Table III. The higher parametric regressions were found to be as good as Eq. 1.

From the aforementioned results and discussion it can be concluded that Eqn. 2 is the best equation for modeling antimalarial activities of the compounds used in the present study.

Table IV

Estimated and observed antimalarial activities (Δ MST) of 2,4-diamino-6-quinazoline sulfonamide derivatives using regression expression 2

Compd. No	Δ MST		Residual
	Observed	Estimated	
1	0.100	1.099	-0.9900
2	8.700	8.095	0.6050
3	3.300	1.762	1.5375
4	2.300	2.982	-0.6815
5	0.300	-0.146	0.4460
6	0.700	1.886	-1.1863
7	0.100	1.886	-1.7862
8	4.400	3.306	1.0940
9	0.300	0.891	-0.5910
10	4.700	4.167	0.5333
11	2.500	2.113	0.3867
12	1.000	1.652	-0.6522
13	0.200	1.528	-1.3281
14	0.300	-1.125	1.4254
15	0.700	0.735	-0.0347
16	3.100	2.510	0.5895
17	0.300	0.071	0.2294
18	0.300	1.260	-0.9598
19	0.500	-0.015	0.5150
20	0.300	-0.557	0.8571

This correlation is good because it involves lesser number of parameters and is statistically the best amongst all other models that we have tried.

In order to confirm our findings, we have estimated antimalarial activities of the compounds using Eq. 2. These values are shown in Table IV. Table IV also contains the observed antimalarial activities for comparison. The comparison has shown that the estimated values are very close to the observed ones, thus confirming our findings.

Further confirmation in favour of our results is obtained by plotting OMST estimated against the observed ones, wherein an excellent linear correlation was observed (Fig. 2).

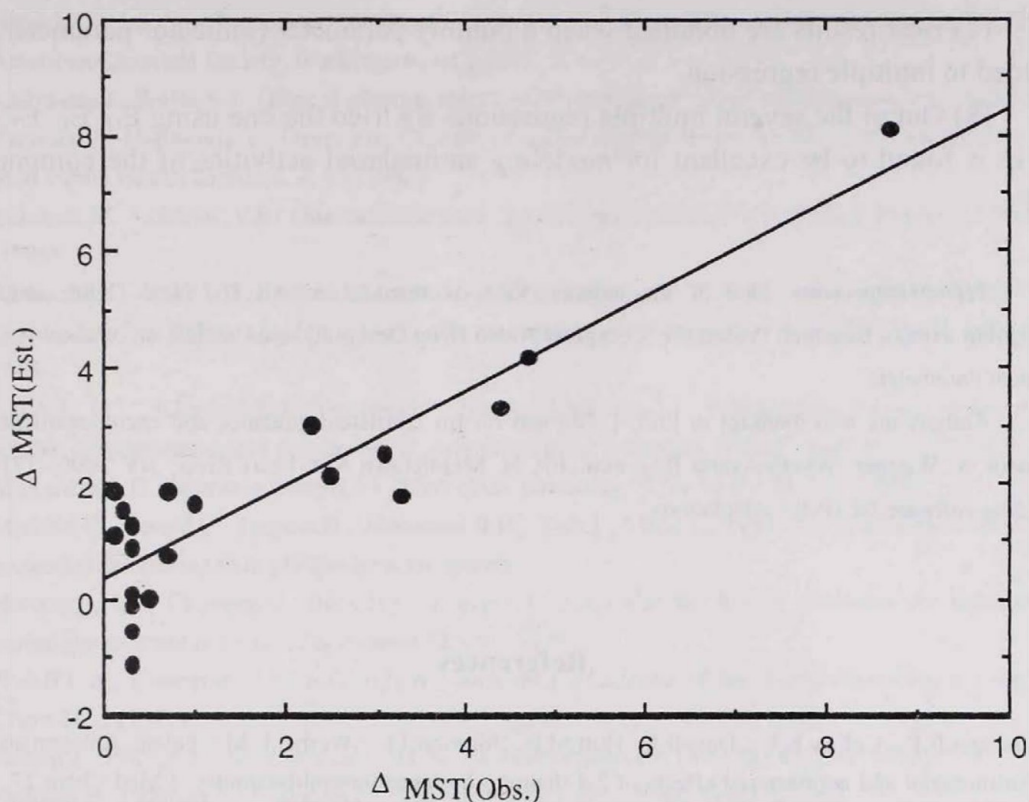


Fig. 2. Correlation of estimated Δ MST with observed Δ MST for 2,4-diamino-6-quinazoline sulfonamide derivatives used in the present study

Conclusions

Based on the results and discussion the following conclusions are made:

- (1) The compounds used have potent antimalarial activity.
- (2) Substitution in the aromatic nucleus changes antimalarial activity.
- (3) No statistically approved correlation exists between antimalarial activities and substituents in the aromatic nucleus.
- (4) Degeneracy is observed both in antimalarial activity as well as correlating parameters used.
- (5) No univariate correlations between activity and any of the correlating parameters exist. Thus, no single parameter is capable of modeling the antimalarial activities of the compounds used.
- (6) Slightly better results are obtained when the data is subjected to multiple regression analysis.

(7) Best results are obtained when a dummy parameter (indicator parameter, I_p) is added to multiple regression.

(8) Out of the several multiple regressions we tried the one using E_H , E_L , E_T , CD and I_p is found to be excellent for modeling antimalarial activities of the compounds used.

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PHYTOTOXICITY OF PATHOGENIC FUNGI AND THEIR MYCOTOXINS TO CEREAL SEEDLING VIABILITY

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Aspergillus flavus, *Alternaria alternata* and *Fusarium oxysporum* were found to be the pathogenic fungi mostly reducing cereal (barley, sorghum and wheat) seedlings. The pathogens have the ability to produce aflatoxin B₁ and G₁, diacetoxyscirpenol, kojic acid and tenuazonic acid that reduced seedling viability. The inhibition dose for 50% reduction (LD₅₀) was recorded by aflatoxins at 0.83 mg L⁻¹ for barley, 1.74 mg L⁻¹ for wheat and 2.75 mg L⁻¹ for sorghum. Diacetoxyscirpenol produced its inhibition at 1.26 mg L⁻¹ for barley, 3.98 mg L⁻¹ for wheat and 10 mg L⁻¹ for sorghum. Kojic acid induced 50% inhibition at 63 mg L⁻¹ for barley, 105 mg L⁻¹ for wheat and 251 mg L⁻¹ for sorghum. However, tenuazonic acid was less toxic where, the toxicity was ranged between 79–550 mg L⁻¹. The inhibition in germination was more pronounced in barley followed by wheat and negligible in sorghum to all tested mycotoxins. This inhibition attributed to the reduction in seedling amylase activity. Amylase was also reduced in the same trend: barley > wheat > sorghum. Grain treatment with carboxin-captan and thiophanatemethyl-thiram at 1g kg⁻¹ grain increased seedlings vigour of wheat in sterilized soil by 45 and 22%, barley by 24 and 33% and sorghum by 15 and 30%, respectively. These fungicides have also a positive effect on cereal when soil was inoculated with *A. flavus*, *A. alternata* and *F. oxysporum*.

Keywords: phytotoxicity of *Aspergillus flavus*, *Alternaria alternata*, *Fusarium oxysporum* aflatoxin, diacetoxyscirpenol, kojic acid, tenuazonic acid

One of the major factors in the low productivity of seeds is poor seed germination and early seedling mortality due to seed-borne fungi. Several grain-borne fungi, including species of *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium* have been implicated as seedling pathogens of cereals [1–4]. Since the discovery of aflatoxin in 1960s a number of reviews appeared regarding the biological effects of aflatoxin and other mycotoxins on poultry and farm animals. Comparatively few records are available with regard to their adverse effects on plant [5, 6].

Fungicides are routinely applied to control seed-borne fungi at storage [7–12]. However, there is little information on cereal seedling diseases and their control.

We isolated and screened fungi pathogenic to cereal (barley, sorghum and wheat) seedlings. Also, the phytotoxicity of their mycotoxins to cereal viability and amylase activity was studied. The role of application of fungicides to grains was also investigated on both infected and uninfected soil.

Materials and methods

Potential pathogenicity of fungal isolates to seedlings

Fungi were isolated from cereal seedlings that had not emerged from the soil. The seedlings were washed and sterilized for 5 min in a solution of 2% NaOCl and planted on PDA. Plates were incubated at 30 °C and fungi growing from seedling tissues were identified and used in cereal infestation. Furthermore, the pathogenic fungi as *Fusarium oxysporum* was also isolated from root rot on collected plants in the field.

Cereal infestation method

The common pathogenic fungal species were assessed for pathogenicity to barley (*Hordeum vulgare* L.), sorghum (*Sorghum vulgare* Pers) and wheat (*Triticum aestivum* L.) seedlings. Each isolate was inoculated into a liter flask containing 200 g of a sterilized mixture of river sand, coarse whole-meal wheat-flour, and water in the ratio of 100: 2: 26. These cultures were incubated at 30 °C for 2 weeks. Plastic pots (9 cm diameter) were half-filled with soil and each was sown with 15 grains. Grains were then covered with a 1 cm layer of an isolate culture or sterile sand of whole-meal medium for control, followed by 1 cm layer of sand. The pots in triplicate were irrigated by tap water enough to keep the water content in soil at 60% of the holding capacity. Containers were then placed in incubator at 22 °C for 7 days. Germination index (GI) and seedling-vigour index (SVI) was assayed. Germination index was calculated by the following formula:

$$GI = \frac{\text{Total number of grains germinated}}{\text{Total number of grains observed}} \times 100$$

Seedling-vigour index was calculated by multiplication the shoot length (cm) in GI.

Grain imbibition in mycotoxin

The grains were soaked in different concentrations of aflatoxin B₁ and G₁ (2:1), diacetoxyscirpenol, kojic acid and tenuazonic acid for 10 h. Control grains were soaked in sterile distilled water. The soaked grains were then placed on moist paper and kept for germination in Petri dishes at 25 °C. Triplicate dishes were used for each treatment and each contained 15 grains. The grains were studied after 2–5 days of imbibition for the emergence of radicle and plumule. SVI was calculated by multiplication the GI in the mean length of plumule and radicle. Standard toxins were purchased from Sigma Company and prepared in our laboratory by fermentation with *A. parasiticus* IMI 120920, *F. tricinctum* (Z 90-410) and *A. alternata* IMI 89344.

Amylase activity determination

The activity of amylase in embryo of grains was demonstrated after 2–5 days of imbibition. The seedlings were frozen and the grains in triplicate were separated and bisected transversely and placed with embryo cut ends downwards on 1% agar containing 1% soluble starch. After incubation at 25 °C for 1 h the half-grains were removed and the plates were flooded with an iodine solution. The diameters of the non-stained zones in the blue background indicated the extents of amylase activity.

Effect of grain treatments on seedling vigour improvement

Barley, sorghum and wheat grains were treated with fungicides listed in Table I. Each formulation was applied at three different doses (1, 3 and 5 g kg⁻¹ grain). Plastic pots were half-filled with soil and seeded with 15 fungicide-treated grains. Grains were then covered with a 2 cm layer of sand. The pots were irrigated by tap water enough to keep the water content in soil at 60% of the holding capacity. Pots in triplicate for each treatment and control were incubated at 22 °C for 7 days. SVI was assayed for each treatment by multiplication the shoot length in GI.

Effect of grain treatments on pathogenicity of individual pathogens

A test was carried out to determine if the seedling improvement fungicides could protect them from pathogenicity of *A. alternata*, *A. flavus* and *F. oxysporum*. The methods described above were applied.

Statistical analysis of the results

Triplicate data of each experiment were analyzed statistically using a program of one-way analysis of variance (PC-state computer program). Probit analysis was

conducted to determine the concentration of mycotoxins inducing 50% (LD₅₀) inhibition in the viability of cereals' grains [6].

Table I

Fungicides used^a

Trade name	Formulation	Common name (active ingredients)	Manufacturer
Homai 80	80 WP	Thiophanatemethyl (50%) - Thiram (30%)	Nippon Soda
Monceren-combi	70 DS	Pencycuron (20%) - Captan (50%)	Bayer
Rizolex-T	50 WP	Tolclofomethyl (20%) - Thiram (30%)	Sumitomo
Vitavax 300	75 WP	Carboxin (37.5%) - Captan (37.5%)	Uniroyal

^aWP= wettable powder; DS= dry seed-dressing

Results and discussion

Effect of fungal species on seedling viability. The common fungal forms frequently associated with the barley, wheat and sorghum grains were screened for ability to induce mortality using a grain infestation method. The pathogenic fungi induced variable reduction in seedling viability: *Aspergillus flavus* (47%), *Alternaria alternata* (40%), *Fusarium oxysporum* (38%), *Rhizopus stolonifer* (31%), *A. niger* (30%), *A. fumigatus* (13%) and *Penicillium corylophilum* (5%) (Table II). *A. alternata*, *A. flavus* and *F. oxysporum* were the most commonly isolated fungi capable of killing or reducing cereal seedlings and have the ability to produce their respective toxins. Grain-borne fungi by producing some toxins may injure the embryo resulting in germination failures or in case of germination may reduce seedling vigour. Furthermore, mycotoxin contaminated commodities have been from time to time returned to agricultural soils. If toxin is not rapidly degraded by the soil microflora, it may be absorbed by the root system of crops and translocated to the foliage and developing fruits [13, 14]. Accumulation of mycotoxin by plants could not only represent a health hazard to the consumer, but may also seriously affect the productivity of the plants.

Table II

Mean seedling vigour (cm) of grains sown in soil containing cultures of different fungal species^a

<i>Rhizopus stolonifer</i>	<i>Penicillium corylophilum</i>	<i>Fusarium oxysporum</i>	<i>A. niger</i>	<i>A. fumigatus</i>	<i>Aspergillus flavus</i>	<i>Alternaria alternata</i>	Control	Grains
940	1220	980	860	1060	840	760	1240	Barley
890	1200	740	840	1180	440	720	1300	Sorghum
860	1270	700	1000	1140	780	880	1360	Wheat
31	5	38	30	13	47	40	–	% Seedling inhibition
–	–	Diacetoxyscirpenol	–	–	Aflatoxin B ₁ &G ₁ , kojic acid	Tenuazonic acid	–	Mycotoxins potentiality

^a Data represent the mean of three replicates

Table III

Effect of mycotoxins on cereal grains germination after 5 days of incubation at 25 °C^a

Mycotoxin	Conc (mg L ⁻¹)	Barley					Sorghum					Wheat				
		P	R	GI	Seedling viability		P	R	GI	Seedling viability		P	R	GI	Seedling viability	
					SVI	Inh %				SVI	Inh %				SVI	Inh %
Control	0	20	50	97	679	-	21	35	100	560	-	21	51	100	720	-
Aflatoxins B1 & G1 (2:1)	0.5	20	45	92	598	12	20	33	100	530	5.4	20	45	97	631	12.4
	1	19	32	49	250	63	18	30	100	480	14.3	16	30	92	423	41.3
	5	0	0	0	0	100	10	8	100	180	67.9	7	15	86	189	73.8
	10	0	0	0	0	100	4	4	82	66	88.2	4	3	48	34	95.3
Diaceoxy- scirpenol	1	20	30	93	465	31.5	13	29	100	470	16.1	2	37	97	553	23.2
	5	9	21	86	258	62	10	24	100	340	39.3	10	21	96	298	58.6
	10	4	8	32	38	94.5	9	14	100	230	58.9	9	17	96	250	65.3
Kojic acid	50	20	34	97	524	22.8	21	34	100	550	1.8	21	31	100	520	27.8
	100	5	18	10	23	96.6	18	21	100	390	30.4	13	19	88	282	60.8
	500	0	0	0	0	100	10	5	91	137	75.5	9	8	87	148	79.4
	1000	0	0	0	0	100	4	2	80	48	91.4	4	1	70	35	95.1
Tenuazonic acid	50	15	34	89	436	35.8	19	35	100	540	3.6	20	42	100	620	13.9
	100	8	30	68	258	62	16	30	100	460	17.9	11	35	97	446	38.0
	500	7	24	19	59	91.3	13	27	100	410	26.8	13	33	87	400	44.4
	1000	0	0	0	0	100	7	13	100	200	64.3	6	17	77	177	75.4

^aP= plumule (mm length); R= radicle (mm length); GI= germination index; SVI =seedling-vigour index (cm). Data represent the mean of three replicates.

Effect of mycotoxins on seedling viability. The most pathogenic fungal species *A. alternata*, *A. flavus* and *F. oxysporum* have the ability to produce the toxins; aflatoxin B₁ and G₁ and kojic acid by *A. flavus*, tenuazonic acid by *A. alternata* and diacetoxyscirpenol by *F. oxysporum* (Table II). Aflatoxin reduced the radicle lengths, germination index (GI) and hence the seedling viability of barely at 1 mg L⁻¹ and sorghum at 5 mg L⁻¹ (Table III). Germination index of barley was more sensitive than that of wheat, but sorghum was not affected. The inhibition in shoot lengths were comparatively lesser than the length of root. Probit analysis of the data gave the values of inhibition dose for 50% reduction (LD₅₀) in the grain viability. LD₅₀ value was for barley 832 µg L⁻¹ and for sorghum 2754 µg L⁻¹. In this respect, aflatoxin was reported to inhibit the germination and root elongation in cotton and soybean [15–17]. Sinha and Sinha [6] found that maximum inhibition in GI of wheat was observed at 2000 µg L⁻¹ aflatoxin B₁.

Seedling viability in different cereal grains treated with 5 mg L⁻¹ diacetoxyscirpenol was inhibited by about 62% in barley, 58% in wheat and 39% in sorghum. LD₅₀ value was for barley 1.26 mg L⁻¹ and for sorghum 10 mg L⁻¹. Kojic acid induced 50% inhibition in seedling viability of barley at 63 mg L⁻¹, wheat at 105 mg L⁻¹ and sorghum at 251 mg L⁻¹. Tenuazonic acid induced 50% inhibition in seedling viability of barley at 79 mg L⁻¹, wheat at 316 mg L⁻¹ and sorghum at 550 mg L⁻¹. The inhibition of grain emergence and seedling viability was more pronounced in barley followed by wheat and it was negligible in sorghum to all tested mycotoxins

Effect of mycotoxins on seedling amylase activity. Mycotoxins affect amylase activity, causing inhibition of starch (major food reserve) hydrolysis and consequent unavailability of sucrose (utilisable substrate) to the embryonic axis during the period of imbibition. This inhibition was more pronounced in barley followed by wheat grains and directly influenced by the concentration of toxins (Table IV). Previously, Chatterjee [18] indicated that amylase activity was inhibited in maize grain infected by *A. parasiticus*. Aflatoxin B₁ inhibits protein and nucleic acid synthesis in germinating seeds of mung and maize [19, 20].

Effect of fungicides on seedling viability. Formulations and rates of application of carboxin-captan, pencycuron-captan, thiophanatemethyl-thiram and tolclofosmethyl-thiram were determined (Figure 1A). The results showed that thiophanatemethyl-thiram and carboxin-captan, increased seedling viability at all application rates (1, 3 and 5 g kg⁻¹) in sterilized soil. However, different treatments of pencycuron-captan and tolclofosmethyl-thiram lead to the development of moderate and high level of fungitoxicity in young sorghum seedlings.

Table IV

Effect of mycotoxins on amylase activity of cereal grains after 3 days of imbibition at 25 °C^a

Mycotoxins	Conc. (mg L ⁻¹)	Amylase activity (%)		
		Barley	Sorghum	Wheat
Control	0	100	100	100
Aflatoxins	0.5	87	87	90
B1 & G1 (2:1)	1	75	75	80
	5	37	62	50
	10	25	50	40
Diacetoxyscirpenol	1	60	75	60
	5	40	62	45
	10	35	60	40
Kojic acid	50	70	100	90
	100	60	100	80
	500	55	87	60
	1000	45	80	50
Tenuazonic acid	50	70	87	90
	100	65	85	80
	500	50	75	70
	1000	45	70	50

^aData represent the mean of three replicates

The effects of selected carboxin-captan, pencycuron-captan and thiophanatemethyl-thiram on seedling viability in soil heavily inoculated with *A. alternata*, *A. flavus* and *F. oxysporum* were studied separately. The low rate (1g kg⁻¹) of carboxin-captan and thiophanatemethyl-thiram applied to sorghum grains and the medium rate (3g kg⁻¹) of pencycuron-captan applied to barley gave high stands under *A. alternata* compared to the untreated grains (Figure 1B). Previously, Kumar and Patnaik [21] found that 2g kg⁻¹ of carboxin and captan improved the germination and seedling vigour of pigeon-pea infected with *A. alternata*.

The medium dose of thiophanatemethyl-thiram improved the viability of sorghum seedling infected with *A. flavus* (Figure 1C). Kannaiyan et al. [22] showed that 3 g kg⁻¹ of thiophanatemethyl controlled *A. flavus* in groundnut seed and improved crop stand.

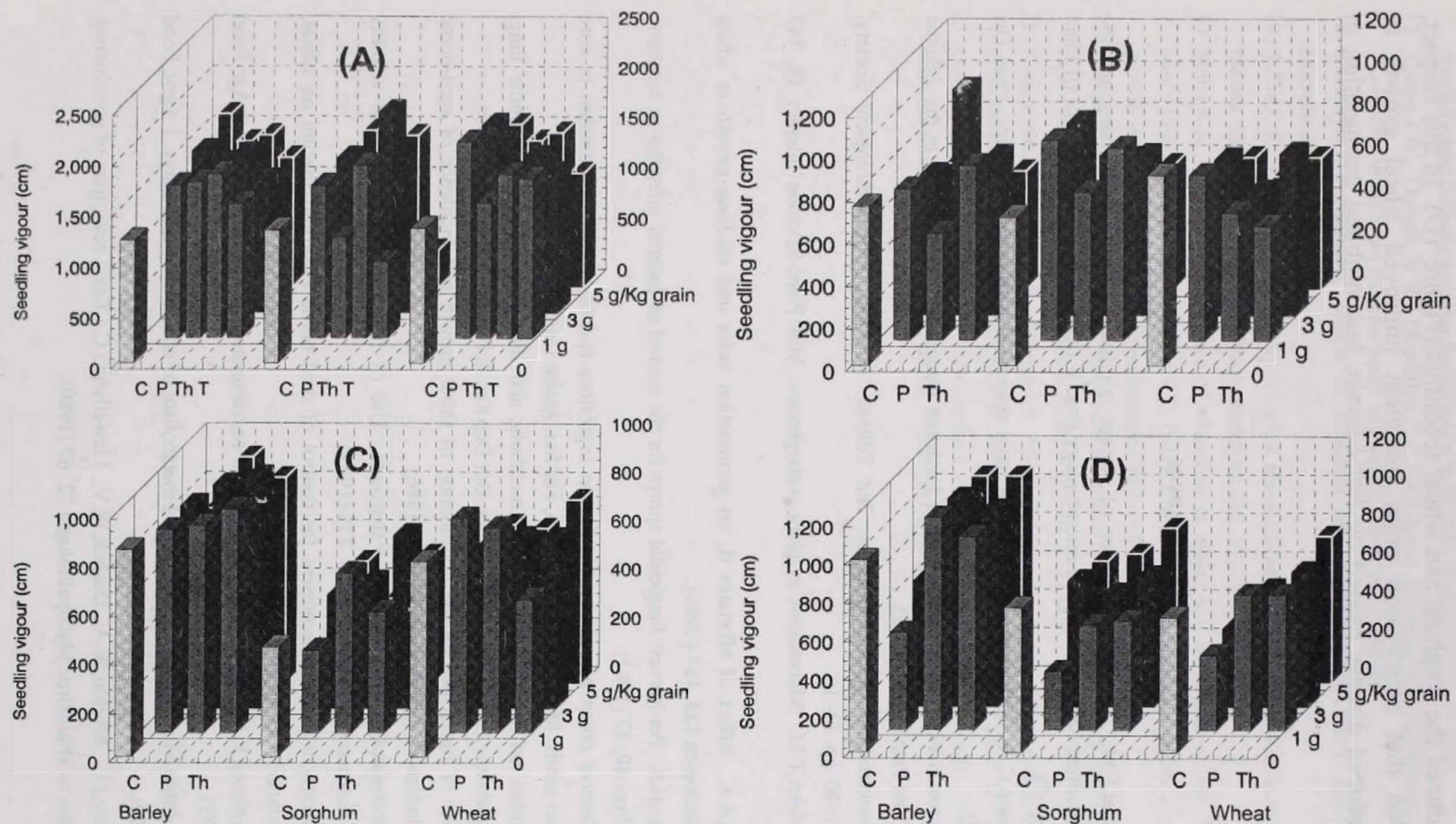


Fig. 1. Mean seedling vigour (cm) of grain treated with fungicides and sown in sterilized soil (A) or soil containing culture of *A. alternata* (B); *A. flavus* (C); *F. oxysporum* (D).

C = carboxin-captan; P = pencycuron-captan; Th = thiophanatemethyl-thiram; T = tolclofosmethyl-thiram.

All doses of penicuron-captan increased the viability of barley infected with *F. oxysporum*, but reduced the sorghum and wheat seedlings (Figure 1D). In this respect, Duffy [23] showed that penicuron grain treatment improved wheat growth in greenhouse and reduced disease caused by *Rhizoctonia*, but inhibited germination *in vitro*.

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XYLANOLYTIC ACTIVITIES OF *STREPTOMYCES* SP. 1 – TAXONOMY, PRODUCTION, PARTIAL PURIFICATION AND UTILIZATION OF AGRICULTURAL WASTES

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Twenty-four different strains of *Streptomyces* spp. isolated from Egyptian soil were tested for their ability to produce extracellular xylanases. Of all these isolates a *Streptomyces* sp. that had the highest potential for xylanolytic activity was chosen. From various morphological, physiological and antagonistic properties, this isolate was found to belong to *Streptomyces lividans*. Factors affecting xylanase production by this organism in a basal salt medium containing purified sugar-cane bagasse xylan as a sole carbon source were examined. A noticeable increase in enzyme activity was observed in the presence of peptone or soyabean meal. However, a slight increase was noticed with ammonium sulphate. Optimum production for xylanase was achieved after five days incubation on a rotary shaker (180 rpm) at 30 °C. The initial pH values were around neutrality. In addition, this organism has high potential for xylanolytic activity when grown on lignocellulosic wastes including corn cobs, wheat bran, peanut shells, sawdust, wheat straw and sugar-cane-bagasse. Partial purification of the enzyme in the culture supernatant was achieved by salting out at 50–80% ammonium sulphate saturation with a purification of 9.03-fold and 57.9% recovery.

Keywords: *Streptomyces*, xylanase, agricultural wastes, biodegradation, hemicellulase

Hemicellulases from microbial sources have gained great attention for utilization and converting hemicellulosic residues to fermentable useful products [1]. These can be used in many fields of human nutrition and also for cosmetics [2].

Xylanases have been found in various organisms, including bacteria, fungi, insects, snails and plants. Fungal xylanases have received great attention [1, 3–9]. Cellulase-free xylanase has been found in a novel east strain by Bastawde et al. [10]. Xylanase was also produced by bacteria was described [11–13]. Slight attention was

given to the xylan degrading enzymes of Actinomycetes. [14–17]. Recently, xylanases were used in pulp bleaching for paper production [18].

In this work we tried to select a *Streptomyces* sp. capable of producing a highly active extracellular xylanase for utilizing sugar-cane bagasse xylan. We describe the taxonomical characters of this organism, the optimal culture conditions for xylanase production, and partial purification of the enzyme preparation. Utilization of various agricultural wastes by xylan-degrading enzyme(s) of the selected *Streptomyces* sp. was also studied.

This will make feasible the production of xylanase as well as formation of fermentable useful products. Also, this will lead to better utilization of local huge amounts of wastes as hemicellulosic residues.

Materials and methods

Microorganisms

Twenty-four *Streptomyces* isolates were screened for their xylanolytic activity. They were isolated from Egyptian soil in different localities of the Nile Delta. The most active one has been taxonomically characterized by the method of the International Streptomyces Project (ISP) recommended by Shirling and Gottlieb [19–22] and Krassilnikov et al. [23].

The antagonistic properties of this isolate were monitored by the agar diffusion method [24] on nutrient agar media for bacteria (*Bacillus cereus*, *Bacillus subtilis*, *Bacillus mucoid*, *Staphylococcus aureus* and *E. coli*) and yeasts (*Candida albicans*, *Candida pseudotropicalis*, *Rhodotorula* sp., *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*) and on Czapeck's dox agar media for filamentous fungi (*Diplodia ohryzae*, *Macrophomina phaseoli*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Aspergillus niger* and *Aspergillus flavus*). All the tested microorganisms are from the culture collection of N.R.C. The antimicrobial activity was observed after 24 h incubation for bacteria or 48 h incubation for yeasts and fungi at 30 °C.

Culture media

For the screening of xylanolytic organisms and the studies on the production of xylanolytic activity a modification of basal salt medium (B.S.M.) of Waksman [25] was used. It contained (g/L): 2.0, KNO₃; 1.0, K₂HPO₄; 0.5, MgSO₄; 0.5, NaCl; 3.0, CaCO₃, 0.01 FeSO₄ and 10.0 xylan (as a sole carbon source). The medium was adjusted to pH 7.0 before autoclaving. A stock culture of *Streptomyces* spp. was

propagated on agar slants with the same constituents. Xylan was replaced by some common agricultural wastes used as substrates for the enzyme production.

Preparation of xylan

Purified xylan was prepared from sugar-cane bagasse according to the method reported by Chen and Anderson [26] and was used as a substrate in the fermentation media for enzyme production.

Screening of xylanolytic organisms

The *Streptomyces* strains were screened for their xylanolytic activity by streaking on agar slants of basal salt medium (B.S.M) containing 1% xylan as a sole carbon source. The positive cultures were chosen to prepare spore suspensions as inoculum for the culture medium.

Cultivation of organism and preparation of crude enzyme

Experiments were carried out in shaken cultures to determine the physiological and physical conditions that would affect the xylanolytic activity of the organism. In all experiments Erlenmeyer flasks (250 ml) containing 50 ml of the appropriate B.S.M. and 1% xylan as a sole carbon source were inoculated with 2% v/v of the spore suspension of the studied organism and incubated at 30 °C on a rotary shaker (180 rpm) for five days. The culture was freed from mycelium debris by centrifugation at 4000 (rpm) under cooling. The supernatant was used as a crude enzyme preparation. Cell growth was determined by measuring the dry cell weight.

Partial purification of xylanase

Fractional precipitation of the crude enzyme preparation was achieved with ammonium sulphate up to 80% saturation, and the solution was left over night. The resulting precipitate of 80% saturation was collected by centrifugation and dissolved in small amount of 0.05 M potassium phosphate buffer pH 6.5 then dialyzed against the same buffer at 5 °C for 24 h. The dialyzed solution obtained was used as the concentrated enzyme solution and designated as "partially purified enzyme preparation".

Enzyme assay

The assay mixture for xylanase activity contained 5 mg of the purified xylan, 2 ml of 0.1 M phosphate buffer pH 6.5 and 1 ml of enzyme preparation. The mixture was incubated for 30 min at 40 °C. Reducing sugars were measured by Somogyi method

[27] using D-xylose as standard. One unit of xylanase activity is defined as the amount of enzyme which release 1 μ M of reducing sugars (equivalent to xylose) per minute under the standard assay conditions.

Protein determination

The protein content was determined according to Lowry et al. [28]. Bovine serum albumin was used as standard.

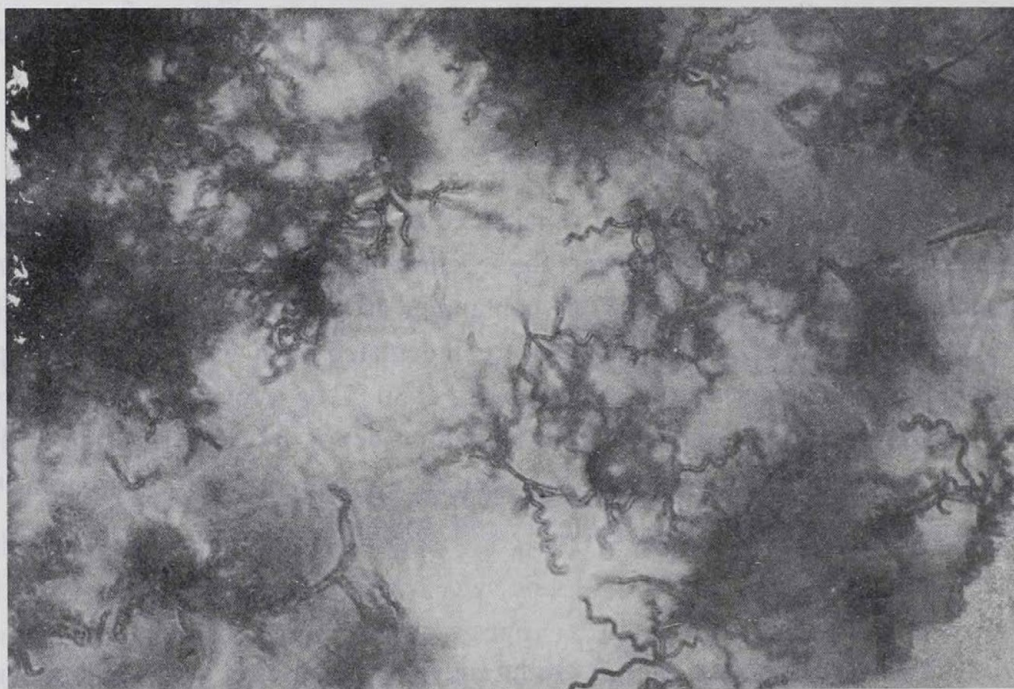


Fig. 1. The spore chains of the isolate ($\times 1600$)

Results and discussion

Taxonomy of xylan-degrading microorganism. From the available cultures of the series of *Streptomyces* spp., a microorganism was selected for its ability to utilize xylan as a sole carbon source. The microscopic examination of the selected strain (Fig. 1) revealed that it produces spiral chains of spores. The spirals were open with 3–7

turns, thus this group is related to the morphological section spira (S). The electron micrographic examination of spore surface showed rod shaped spores with warty surface (Fig. 2).

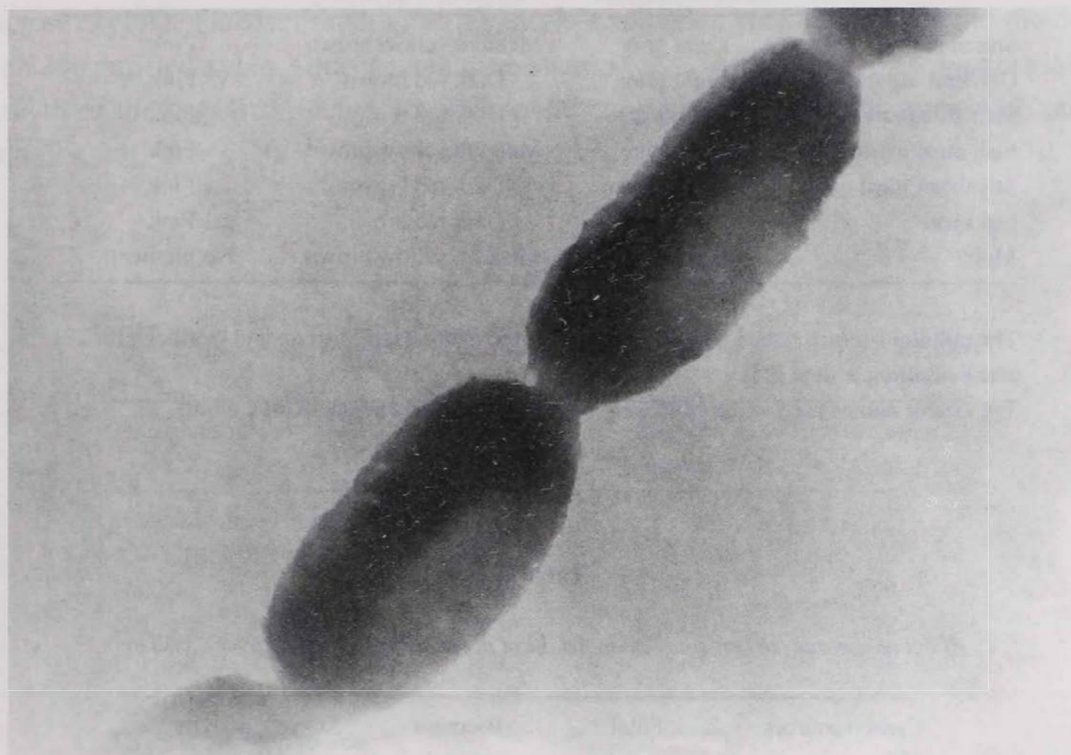


Fig. 2. Scanning electron micrograph of spores of the isolate ($\times 31500$)

Cultural characteristics. The study of cultural properties of the selected isolate on different media at 30 °C for 14 days are shown in Table I. The colour names used in this study were taken from Methuen Handbook of Colour. The aerial mycelium was light gray to gray. The colour of substrate mycelium was dark red brown or medium yellow brown. A pink soluble pigment was produced on most media except molt medium that was not pigmented.

Physiological properties. The isolate does not produce melanoid pigments on peptone-yeast extract-iron agar or tyrosine agar. The assimilation of carbon sources indicated that the selected isolate utilized all different carbon sources as glucose, galactose, fructose, sucrose and arabinose. The organism hydrolyzed starch and failed to produce H_2S .

Table I

Cultural characteristics of 14 days old cultures of the Streptomyces isolate on different media

Name of the media	Colour of		
	Aerial mycelium	Substrate mycelium	Media
Starch nitrate	Light gray	Medium yellow brown	Pink
Glycerol asparagine	Light gray	Dark red brown	Pink
Sucrose nitrate	Medium gray	Dark red brown	Pink
Fish meal extract	Light gray	Medium yellow brown	Pink
Soyabean meal	Light gray	Dark red brown	Pink
Oat meal	Light gray	Dark red brown	Pink
Molt	Light gray	Medium yellow brown	No pigment

The cultural characteristics were determined by the methods of Shirling and Gottlieb [19–22] and Krassilnikov et al [23].

The colour names used in the table were based on Methuen Handbook of Colour.

Table II

Effect of various carbon sources on xylanase production by Streptomyces lividans

Carbon sources 1%	Final pH	Biomass mg/ml	Enzyme activity U/ml
Xylan	8.68	6.8	2.86
Cellulose	8.56	10.5	0.91
Dextrin	8.81	5.6	0.99
Sucrose	8.10	4.0	0.58
Maltose	7.56	6.5	0.58
Xylose	8.11	5.5	0.53
Glucose	7.49	5.7	0.49

Carbon sources with 1% concentration were added to basal salt medium (B.S.M), incubated shaken for 5 days at 30 °C and pH 7.0

Antagonistic properties. The study of the antimicrobial potentialities of the isolate showed that it is active against yeasts but not against bacteria or fungi. Based on the taxonomic properties described above, a comparison of this isolate was made with those published for descriptions of various *Streptomyces* spp. As a result, this selected

isolate was found to belong to the genus *Streptomyces* with close similarities to *Streptomyces lividans* [23].

Induction of xylanase in Streptomyces sp. The response of *Streptomyces lividans* to the addition of various carbon sources indicated that enzyme production was inducible (Table II). Of the carbon sources tested (xylan, cellulose, dextrin, maltose, sucrose, xylose, glucose) the highest xylanase production was obtained in the medium with 1% purified xylan. Glucose, maltose, sucrose or xylose, though they supported the growth of the organism, could not induce the enzyme. These results agree with other data [29, 30].

Table III

Effect of different nitrogen sources on xylanase production by Streptomyces lividans

Nitrogen sources	Final pH	Biomass mg/ml	Enzyme activity U/ml
Inorganic nitrogen source			
KNO ₃ (B.S.M.)	8.66	6.9	2.79
NaNO ₃	8.65	6.2	2.54
NH ₄ NO ₃	7.98	4.0	2.32
(NH ₄) ₂ SO ₄	8.01	5.6	2.68
(NH ₄)H ₂ PO ₄	8.10	5.9	2.56
Organic nitrogen source			
Peptone	9.10	9.5	2.96
Soyabean meal	8.80	8.5	2.93
Casein	8.95	9.6	2.41
Yeast extract	8.75	8.1	3.10

Basal salt medium (B.S.M) supplemented with 1% xylan as sole carbon source and organic or inorganic nitrogen sources (equimolar amounts) incubated shaken for 5 days at 30 °C and pH 7.0

Effect of cultural conditions on xylanase production. To investigate the optimum cultural conditions for xylanase production, the effect of addition of various nitrogen sources on enzyme production was studied. The nitrogen in the B.S.M was replaced by various nitrogen sources with equimolar amounts. As shown in Table III the enzyme activity was not affected by the different inorganic nitrogen sources except by ammonium sulphate, when a slight increase was noticed. An increase in enzyme activity was observed with peptone and soya bean meal as organic nitrogen sources.

Table IV

Effect of xylan concentrations on the production of xylanase
by *Streptomyces lividans*

Xylan concentration % (W/V)	Final pH	Enzyme activity U/ml
0.2	8.50	0.79
0.4	8.53	1.44
0.6	8.61	1.98
0.8	8.61	2.20
1.0	8.68	2.89
2.0	8.80	3.10

Different concentration of xylan were added to basal salt medium (B.S.M), incubated shaken for 5 days at 30 °C

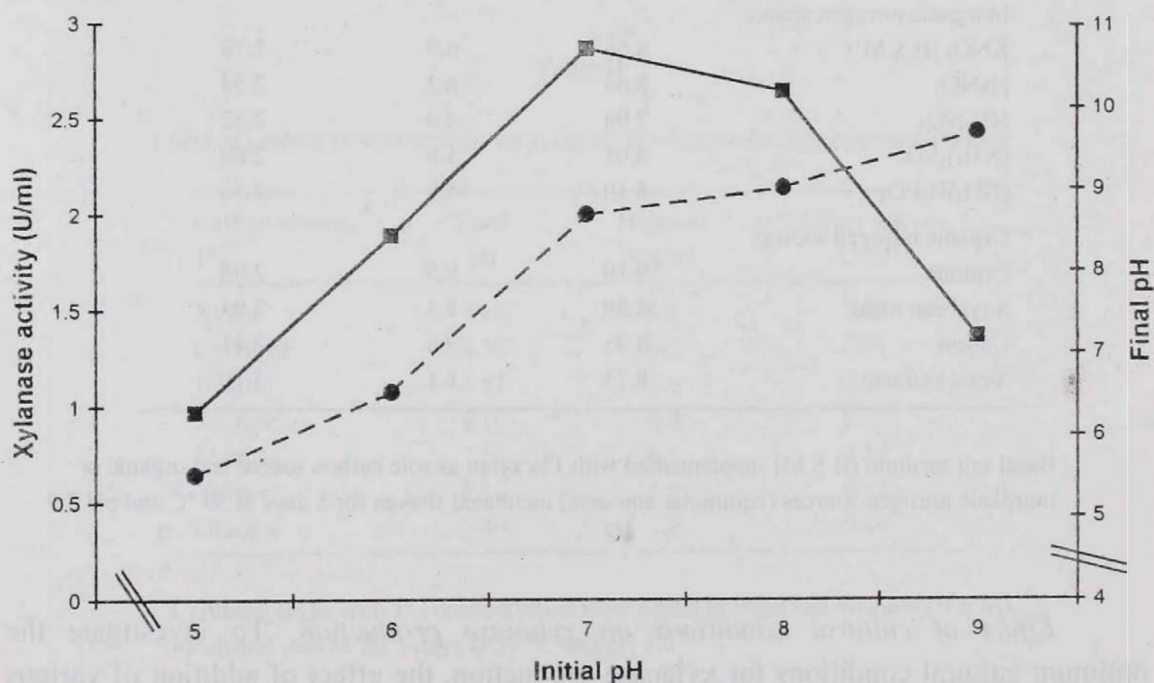


Fig. 3. Effect of initial pH xylanase activity production, shaken culture at 30 °C

—■— xylanase activity and ---●--- final pH

A significant effect of yeast extract on growth was noticed with a slight increase in enzyme production. Thus, we may conclude that yeast extract is a growth factor but not necessary for enzyme production.

Substrate concentrations had a significant effect on the production of xylanase. Increasing the concentration of the substrate (xylan) resulted in a simultaneous increase in the growth of the organism and of xylanase activity of the culture supernatant (Table IV).

Microbial growth and xylanase production were optimal when the culture was incubated at 30 °C. Maximum xylanase activity was observed when the initial pH of the medium was adjusted to 7.0 (Fig. 3). These results agree with data published by Tasuku et al. [14] and McCarthy [31].

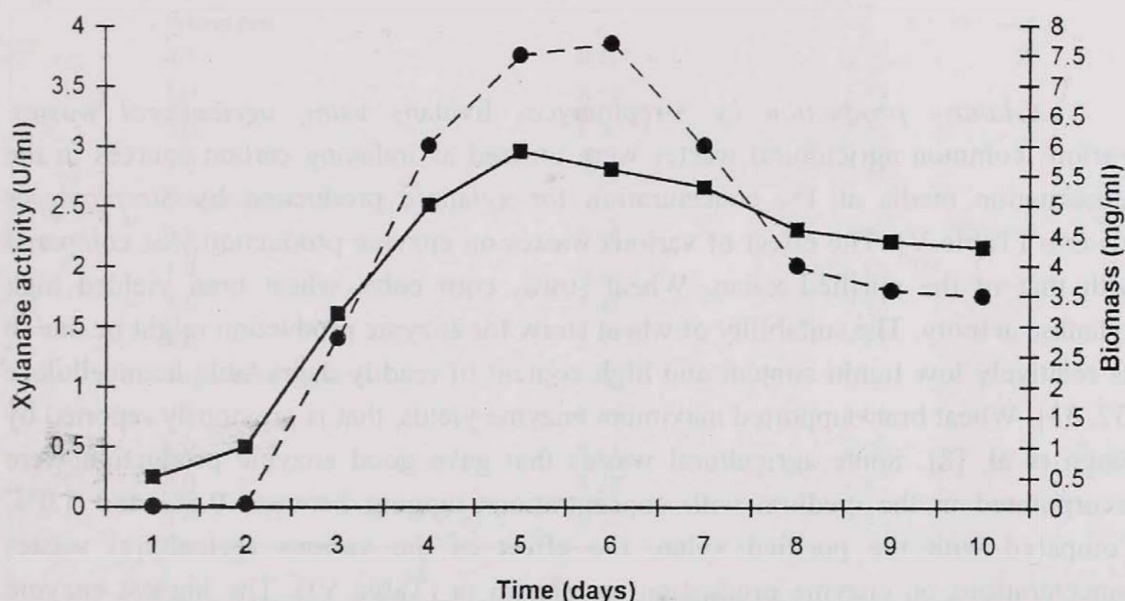


Fig. 4. Time course of xylanase activity production, shaken culture at 30 °C (optimized basal medium)

—■— xylanase activity and ---●--- cell growth (biomass)

Time course of xylanase productivity. By following xylanase production and the organism growth (biomass) (Fig. 4), we conclude that the enzyme production reached a maximum after 5 days of incubation. This was followed by a decline in enzyme activity. The enzyme production was associated with an increase of the growth of the organism.

Table V

Utilization of agricultural wastes by *Streptomyces lividans* for enzyme production

Agricultural wastes	Final pH	Biomass mg/ml	Enzyme activity U/ml
Xylan	8.20	7.5	2.98
Corn cobs	7.40	10.4	3.55
Wheat bran	8.70	9.7	5.19
Peanut shells	8.07	10.7	0.99
Saw dust	7.70	11.8	0.91
Wheat straw	8.20	9.8	6.62
Sugar cane bagasse	8.03	11.0	2.13

Basal salt medium (B.S.M) was supplemented with 1% various agricultural wastes incubated shaken for 5 days at 30 °C

Xylanase production by Streptomyces lividans using agricultural wastes. Various common agricultural wastes were utilized as inducing carbon sources in the fermentation media at 1% concentration for xylanase production by *Streptomyces lividans* (Table V). The effect of various wastes on enzyme production was compared with that of the purified xylan. Wheat straw, corn cobs, wheat bran yielded high xylanase activity. The suitability of wheat straw for enzyme production might be due to its relatively low lignin content and high content of readily degradable hemicellulose [32, 33]. Wheat bran supported maximum enzyme yields, that is previously reported by Singh et al. [8]. Some agricultural wastes that gave good enzyme production were incorporated in the medium with concentrations ranging between 0.5% and 3.0%. Compared with the purified xylan, the effect of the various agricultural wastes concentrations on enzyme production are shown in (Table VI). The highest enzyme yield was obtained with high concentrations of wheat straw, while low concentrations of wheat bran gave good xylanase production. The effect of different concentrations on enzyme yield with sugar-cane bagasse and corn cobs are limited.

These results showed the possibility of using several byproducts as hemicellulosic residues for enzyme production and fermentable useful products that can be used in many fields of nutrition. Also, xylanase enzymes were used in pulp bleaching for paper production [18].

Table VI

The effect of different concentrations of agricultural wastes on xylanase production by Streptomyces lividans

Concentrations of agricultural wastes W/V (%)	Final pH	Enzyme activity U/ml
Xylan		
0.5	8.11	1.79
1.0	8.50	2.90
2.0	8.63	3.22
3.0	9.24	3.61
Corn cobs		
0.5	7.50	3.38
1.0	7.55	3.63
2.0	7.83	4.01
3.0	7.91	4.25
Wheat bran		
0.5	8.10	4.51
1.0	8.50	5.17
2.0	8.70	4.36
3.0	9.0	4.27
Wheat straw		
0.5	8.08	5.24
1.0	8.20	6.86
2.0	8.45	7.96
3.0	8.55	9.18
Sugar-cane bagasse		
0.5	8.14	1.65
1.0	8.21	2.09
2.0	8.28	2.82
3.0	8.30	3.45

Basal salt medium (B.S.M) was supplemented with different concentrations of agricultural wastes ranging between (0.5–3.0%), incubated shaken for 5 days at 30 °C

Partial purification of xylanase from Streptomyces lividans. Attempts to partially purify the enzyme(s) in the culture supernatant with ethanol or acetone proved to be unsatisfactory. Saturation with ammonium sulphate was the most suitable and provided fractions possessing high xylanase activity (Table VII). Salting out with ammonium sulphate up to 50% saturation resulted in a precipitate with low enzymatic activity, therefore this precipitate was discarded. Increasing ammonium sulphate

concentration to 80% afforded a precipitate possessing high xylanase activity with a purification of 9.03-fold and 57.9% recovery of activity (Table VII).

Table VII

Partial purification of the crude xylanase preparation from Streptomyces lividans

Purification steps	Total protein mg	Total units U	Specific activity U/mg	Yield %	Purification fold
Supernatant	620	2856	4.61	100	—
Ammonium sulphate precipitation					
0–30%	199.2	279.3	1.40	9.8	0.30
30–50%	91.8	566.9	6.18	19.5	1.34
50–80%	39.4	1656.4	41.62	57.9	9.03

Thus, ammonium sulphate precipitate (50–80%) showing high xylanolytic activity, and high purification and recovery will be chosen for further purification and characterization studies.

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ENZYMES OF *CANDIDA ALBICANS* CELL-WALL LYTIC SYSTEM PRODUCED BY *STREPTOMYCES THERMODIASTATICUS*

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The production of the enzymes of *Candida albicans* cell-wall lytic system by *S. thermodiastaticus* was found to be affected by some growth conditions and nutritional factors. The highest lytic activity was obtained after 18 h of incubation at pH 5.5 and an incubation temperature of 50 °C. The carbon source influenced the production of the enzymes of the yeast cell wall lytic system. Maximum lytic activity was obtained when *Candida albicans* cell-wall (1 g/100 ml) was used as the sole carbon source. NaNO₃ at 0.1 g/100 ml level was the best nitrogen source for the biosynthesis of the enzymes of the yeast lytic system. From all phosphor sources, microelements, and growth factors tested, KH₂PO₄ (1 g/l), ZnSO₄ (1 mg/l) and Tween 80 (0.1%), respectively were found to favour highest enzymes production of the lytic system. The *Candida albicans* cell-wall lytic system produced by *S. thermodiastaticus* mainly contained chitinolytic and proteolytic activities.

Keywords: *Streptomyces thermodiastaticus*, lytic enzyme, *Candida albicans*, chitinase, protease

Lytic enzymes are produced by various kinds of microorganisms including yeast [1], filamentous fungi [2–4] and bacteria [5–11]. Lytic enzymes have mainly been studied with respect to the biological control of plant pathogenic fungi or protoplast formation from yeast cell and the careful isolation of intracellular proteins [12–13]. Lytic enzymes may also have clinical significance in infectious diseases caused by low-toxic Gram-negative bacteria such as *Serratia marcescens* [14].

Candida albicans is a member of the normal flora of the skin, mouth and the mucous membranes in the respiratory, gastrointestinal, and female genital tracts. In such locations, it may gain dominance and be associated with pathological conditions

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as a secondary invader. Lytic enzymes can thus be used as an agent for treatment of these diseases.

Although the lytic enzymes of mesophilic actinomycetes have been reported by several investigators [12–22], no attempts (as the authors are aware) have been made to purify and study the properties of those produced by thermophilic actinomycetes. In this paper we describe the optimum conditions for the production of the enzymes of *Candida albicans* cell-wall lytic system of a thermophilic bacterium, *Streptomyces thermodiastaticus*.

Materials and methods

Isolation and characterization of the lytic enzymes producing strains

Twenty-four thermophilic actinomycetes strains were isolated from different soil samples, collected from cultivated lands of South Sinei, Egypt, by the dilution plate method on starch-nitrate agar plates supplemented with mycostan (5 µg/ml) as an antifungal agent. The plates were incubated at 50 °C for 3 days and differently looking colonies were picked up. All isolates were purified. The purified isolates were tested for their ability to degrade cell walls of *C. albicans* by point-inoculation onto a basal medium having the following composition (g/l): K₂HPO₄, 0.7; KH₂PO₄, 0.3; MgSO₄·7H₂O, 0.5; FeSO₄·7H₂O, 0.01; ZnSO₄·7H₂O, 0.001 and *C. albicans* cell wall, 10 (wet weight). Plates were incubated at 50 °C for two days and the diameters of the hydrolysis zones around the growth of the cultures were used as an indication of lytic activity. The most active selected isolates were cultured in liquid medium at 50 °C for 18 h with shaking at 150 rpm and the lytic activities of the culture filtrates were assayed. Among the most active isolates isolate No. F was found to produce highest lytic activity in the medium. The bacterial strain was taxonomically characterized according to the keys proposed by International Streptomyces Project [23–24] and Bergey's Manual of Determinative Bacteriology [25]. According to the taxonomical characteristics, the bacterium was identified as a strain belonging to *Streptomyces thermodiastaticus* [26].

Cultivation of lytic enzyme producing Streptomyces strain

Streptomyces thermodiastaticus was grown in 250 ml Erlenmeyer flasks each containing 50 ml of the following medium (g/l): K₂HPO₄, 0.7; KH₂PO₄, 0.3; MgSO₄·7H₂O, 0.5; FeSO₄·7H₂O, 0.01; ZnSO₄·7H₂O, 0.001 and *C. albicans* cell wall, 10 (wet weight). The cell wall employed as carbon and nitrogen source was derived

from a 3-day-old culture of *C. albicans* grown on Sabouraud medium (peptone, 20 g; glucose, 10 g; yeast extract, 5 g; and H₂O, 1 l). Cell walls of *C. albicans* were prepared according to the method described by Northcote and Horne [27]. The pH of the medium was adjusted to 6.8–7.0 and cultures were incubated on a gyratory shaker (New Brunswick Scientific, innova 4330) operated at 150 rpm at 50 °C for 18 h (unless otherwise mentioned). Cells were removed by filtration through Whatman No.3 filter paper. The obtained filtrate was centrifuged at 4000 rpm and the supernatant was filtered through a millipore filter (0.45 µm) and the lytic activity of the cell-free filtrate was determined.

Assay of the lytic activity

Lytic activity was assayed spectrophotometrically (Spectrophotometer, Spectronic, 21 D) by measuring the decrease in turbidity of suspensions of *C. albicans* cell-walls in 0.2 M phosphate buffer (pH 7.0) according to the method adopted by Tominaga and Tsujisaka [28]. One unit of lytic activity was defined as the amount of enzyme which reduces 1% of the initial optical density at 660 nm of the cell wall suspension per 10 min.

Assay of other enzyme activities

Chitinase activity was measured by adding 0.5 ml of enzyme solution to 0.5 ml of colloidal chitin suspended in McIlvaine buffer [29], pH 6.5. After 90 min incubation at 50 °C, the amount of N-acetylglucosamine liberated was determined by the method of Reissig et al. [30]. One unit of chitinase activity was defined as an enzyme quantity which liberated 1 µmol of N-acetylglucosamine per min. Protease activity was assayed according to the method of Anson [31] as modified by Ong and Gaucher [32]. One unit of protease activity was defined as the amount of enzyme which liberates digestion product not precipitated by TCA equivalent to 1 µmol of tyrosine per min at pH 7.0 and incubation at 50 °C.

Protein assay

The protein content was determined according to the method of Lowry et al. [33], using bovine serum albumin as a standard.

Results and discussion

Effect of incubation period. The changes in the enzyme levels and growth yield during 72 hours of incubation of *S. thermodiastaticus* at 50 °C were measured. Figure 1 shows that the highest level of lytic activity, proteolytic activity and biomass yield was obtained after 18 h of incubation. However, two peaks of chitinolytic activity with maximum values were reached at 18 and 66 h of incubation. Maximum chitinolytic activity of *S. thermoviolaceus* was obtained after 24 h of incubation [34]. This difference may be attributed either to the conditions of cultivation or to species differences.

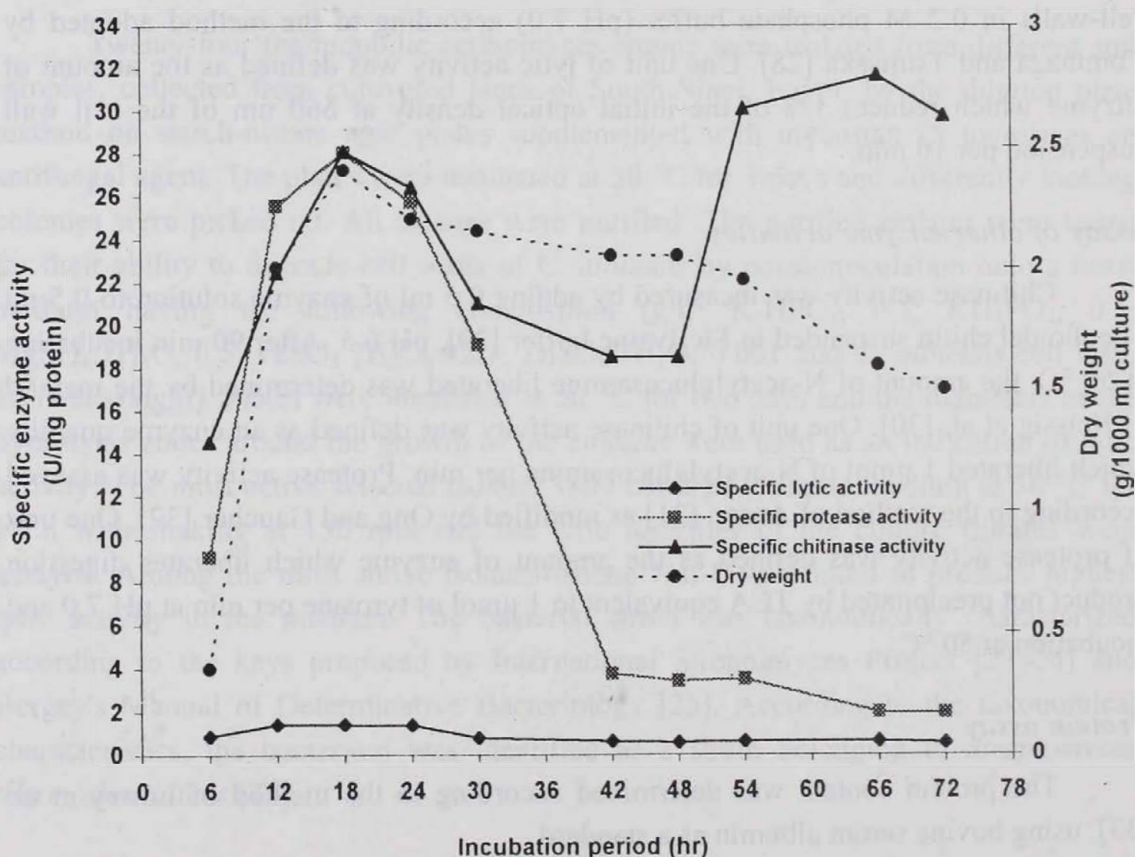


Fig. 1. Effect of incubation periods on *Candida albicans* cell-wall lytic enzyme production and biomass yield of *S. thermodiastaticus*

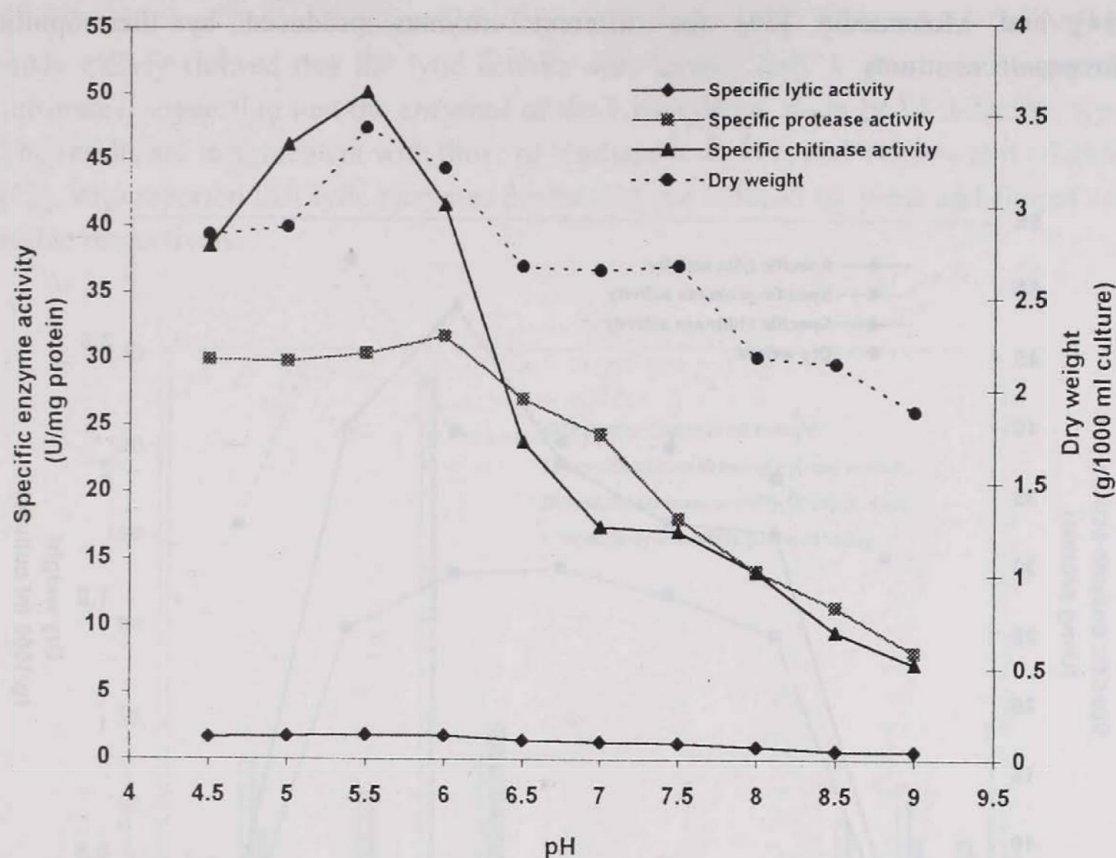


Fig. 2. Effect of pH on *Candida albicans* cell-wall lytic enzyme production and biomass yield of *S. thermodiastaticus*

Effect of pH of the medium. The initial pH value of the medium has a marked effect on growth of the organisms, on the permeability of the cell membrane, as well as on the biosynthesis and stability of the enzymes [35]. Accordingly the effect of the initial pH was studied. The initial pH of the medium was adjusted in the range of 4.5 to 9.0 using diluted HCl or NaOH. The results (Fig. 2) show that *S. thermodiastaticus* exhibited maximum lytic and chitinolytic activities and biomass yield at pH 5.5, while the highest proteolytic activity was obtained at pH 6.0. In this connection Tsujibo et al. [34] reported that *S. thermoviolaceus* produces its highest chitinolytic activity at pH 7.0.

Effect of incubation temperature. *S. thermodiastaticus* was inoculated in the medium at a temperature ranged between 30 to 60 °C. The results in Fig. 3 show that the optimal temperature for maximum enzyme activities was 50 °C, while the optimal temperature for growth was 55 °C. A sharp drop in the enzyme production was observed at 60 °C. These results are in conformity with those reported by Tsujibo et al.

[34] and Mohamedin [36] for different enzymes produced by thermophilic *Streptomyces* strains.

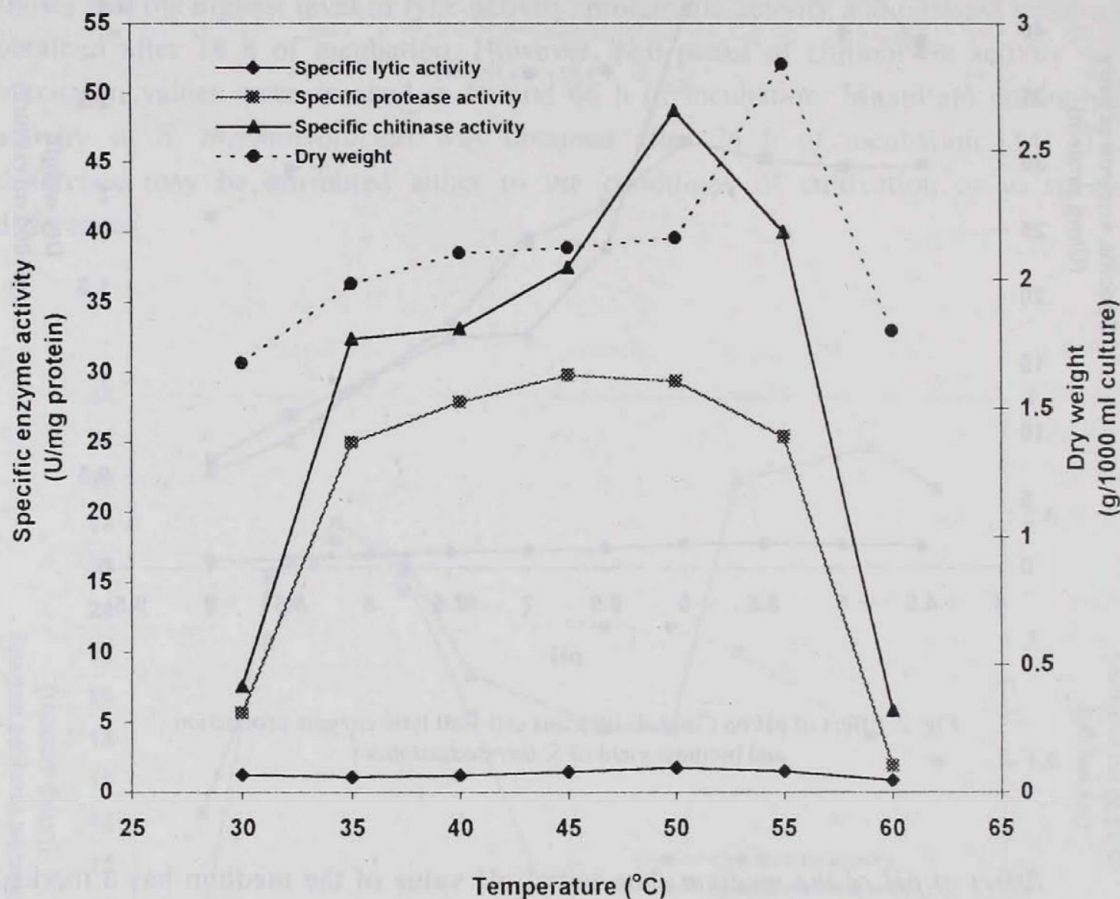


Fig. 3. Effect of temperature on *Candida albicans* cell-wall lytic enzyme production and biomass yield of *S. thermodiastaticus*

Effect of different carbon sources. *C. albicans* cell wall was replaced by different carbon sources at 1% (w/v) to test their influence on the production of the enzymes of the yeast cell-wall lytic system and biomass yield. The results given in Fig. 4 indicate that the highest lytic activity was obtained when the *C. albicans* cell-wall followed by starch and chitin were used as the sole source of carbon. Maximum proteolytic and chitinolytic activities were obtained with mannan and chitin, respectively. Carboxymethyl cellulose inhibits the production of the enzymes of the yeast cell-wall lytic system and biomass yield. The other carbon sources seemed to attenuate production of the enzymes. N-acetylglucosamine followed by the cell wall of

C. albicans was the most favourable for the biomass yield. Meanwhile, the present study clearly showed that the lytic activity was formed only in presence of specific substrates, suggesting that the enzymes of the lytic system might be of inducible type. The results are in agreement with those of Yoshida et al. [13] and Tagawa and Okazaki [22], who reported that lytic enzymes production are induced by yeast and fungal cell walls, respectively.

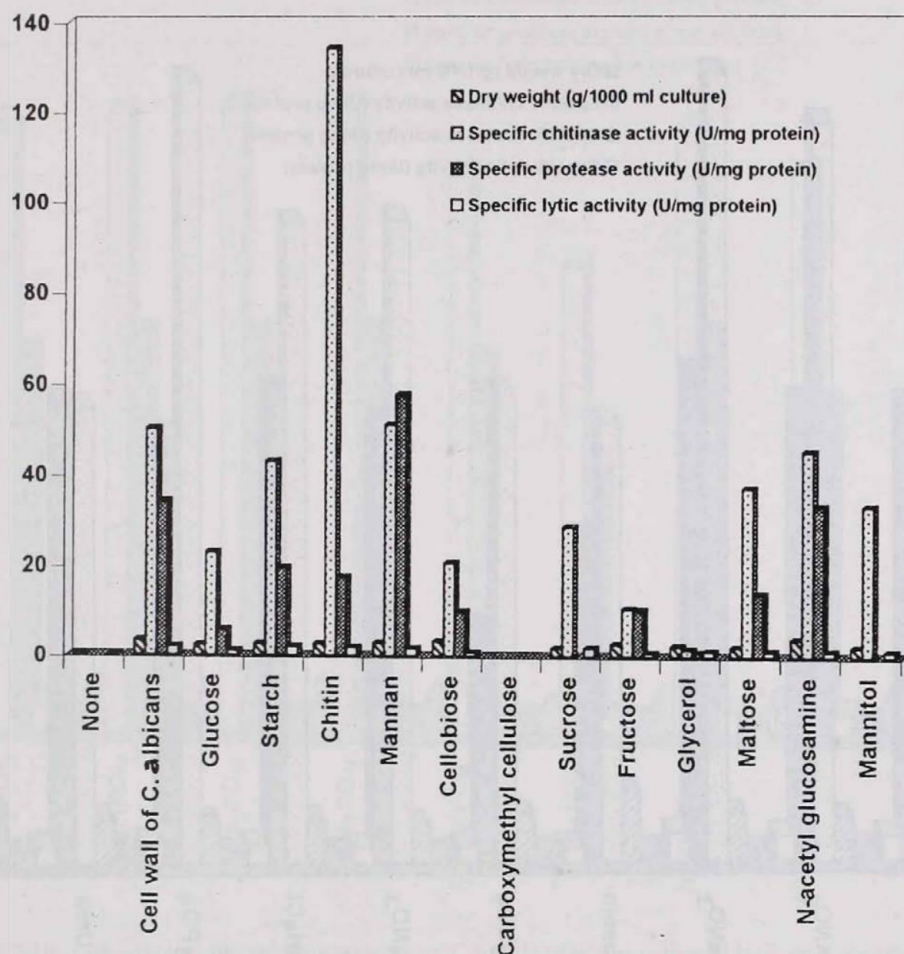


Fig. 4. Effect of different carbon sources on *Candida albicans* cell-wall lytic enzyme production and biomass yield of *S. thermodiastaticus*

Effect of different nitrogen sources. The effect of various organic and inorganic nitrogen sources at 0.1% (w/v) on the production of the enzymes of the yeast lytic system and biomass yield in a medium containing *C. albicans* cell wall, as a sole source of carbon was investigated. The results depicted in Fig. 5 show that the enzyme production and growth of *S. thermodiastaticus* were markedly affected by the nature of

the nitrogen source. Highest lytic, proteolytic and chitinolytic activities occurred with NaNO_3 whereas casein was the best nitrogen source for biomass yield. Gabr et al. [37] have shown that organic nitrogen compounds were superior to inorganic salts for growth and metabolic activities of many *Streptomyces* species. The present results agree with these finding with respect to the superiority of casein for biomass yield. This may be due to the fact that on hydrolysis, the complex organic nitrogen compound (casein) yields a variety of amino acids which might be better for initiating growth.

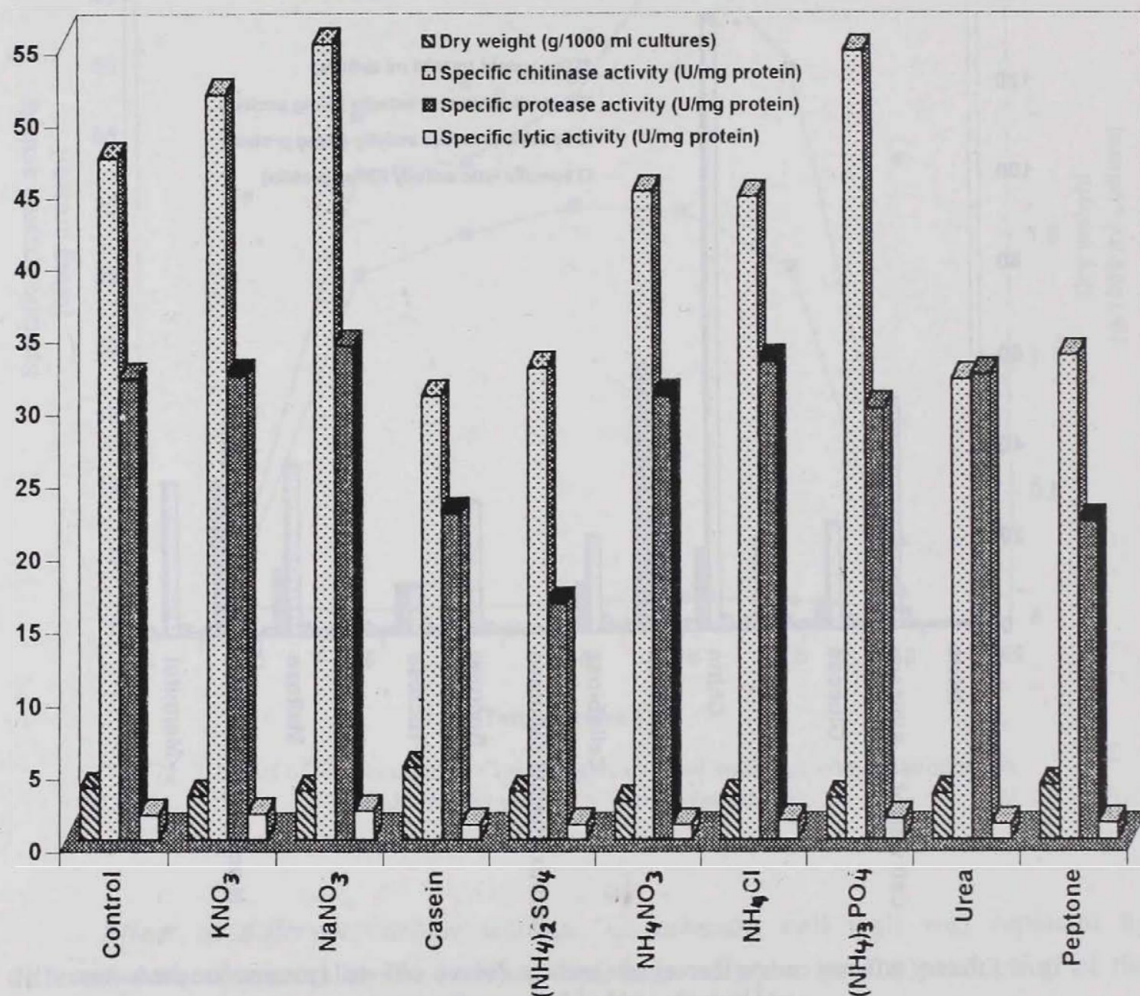


Fig. 5. Effect of different nitrogen sources on *Candida albicans* cell-wall lytic enzyme production and biomass yield of *S. thermodiastaticus*

Effect of different phosphorus sources. The basal medium containing the cell wall of *C. albicans* and NaNO_3 as carbon and nitrogen sources, respectively, were supplemented with mono-, di- and tribasic phosphate sources in a concentration of 1

g/l. Results given in Fig. 6 show that maximum enzyme production and biomass yield was obtained with KH_2PO_4 . This agrees with the results of Skujins et al. [16] and Beyer and Dieckmann [19] who reported that KH_2PO_4 enhanced the lytic activity of the tested *Streptomyces* strains.

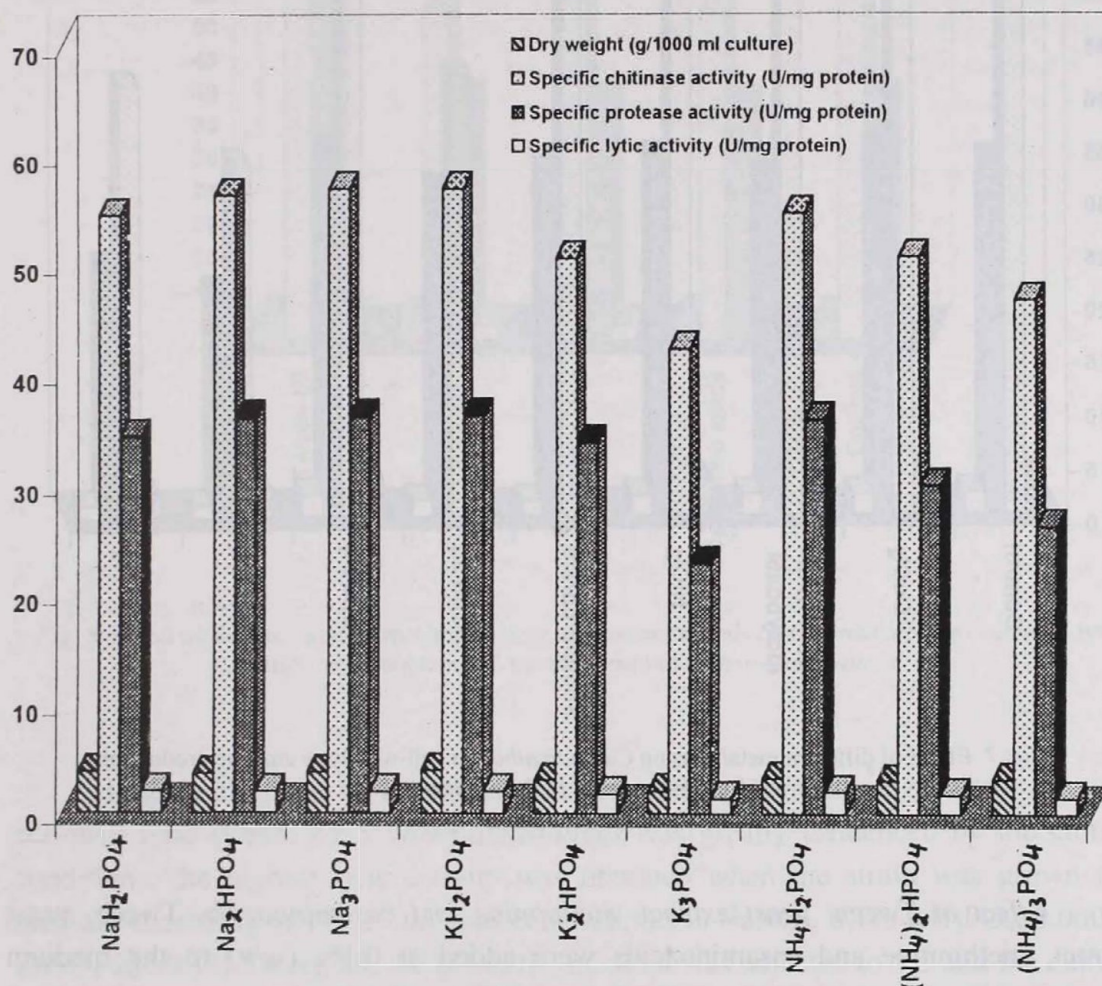


Fig. 6. Effect of different phosphorus sources on *Candida albicans* cell-wall lytic enzyme production and biomass yield of *S. thermodiastaticus*

Effect of different microelements. When various salts of microelements were supplemented at a level of 1 mg/l to the culture medium containing *C. albicans* cell-wall, NaNO_3 , and KH_2PO_4 , it was found that ZnSO_4 favoured production of enzyme activities, whereas ammonium molybdate was the most favourable for growth (Fig. 7). Zinc may act as an activator (co-factor) for enzyme synthesis [37].

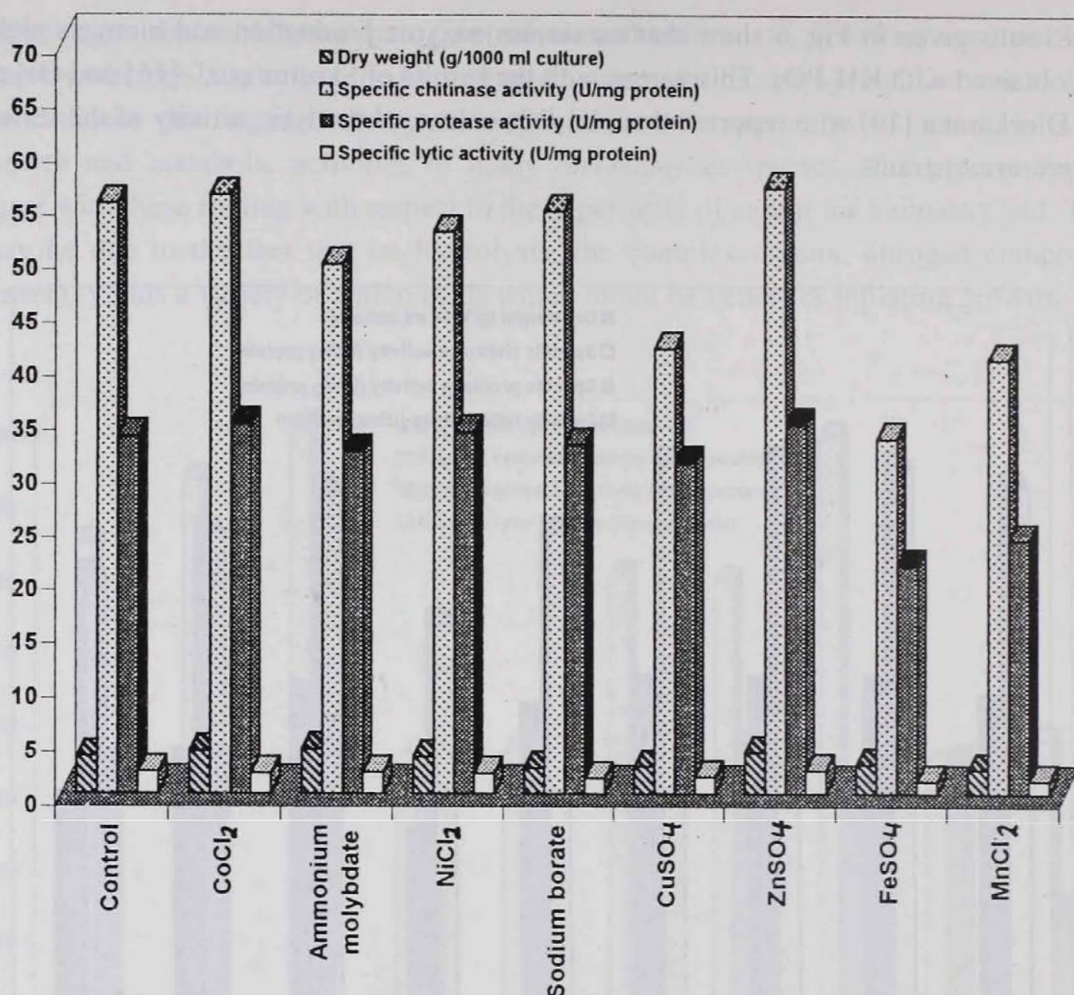


Fig. 7. Effect of different metal ions on *Candida albicans* cell-wall lytic enzyme production and biomass yield of *S. thermophilus*

Effect of Tween, yeast extract, methionine and casaminoacids. Tween, yeast extract, methionine and casaminoacids were added at 0.1% (w/v) to the medium containing *C. albicans*, NaNO₃, KH₂PO₄ and ZnSO₄. The results presented in Fig. 8 show that only Tween 80 enhanced enzyme production of the yeast lytic system and biomass yield. The non-ionic detergent Tween 80 has been reported to enhance the production of several enzymes by a great variety of microorganisms [19, 25, 38, 39]. As a surfactant, Tween 80 may interact with the cell membranes and the surface of insoluble substrates, probably alters the membrane permeability and thus leads to increased release or solubilization of extracellular enzymes.

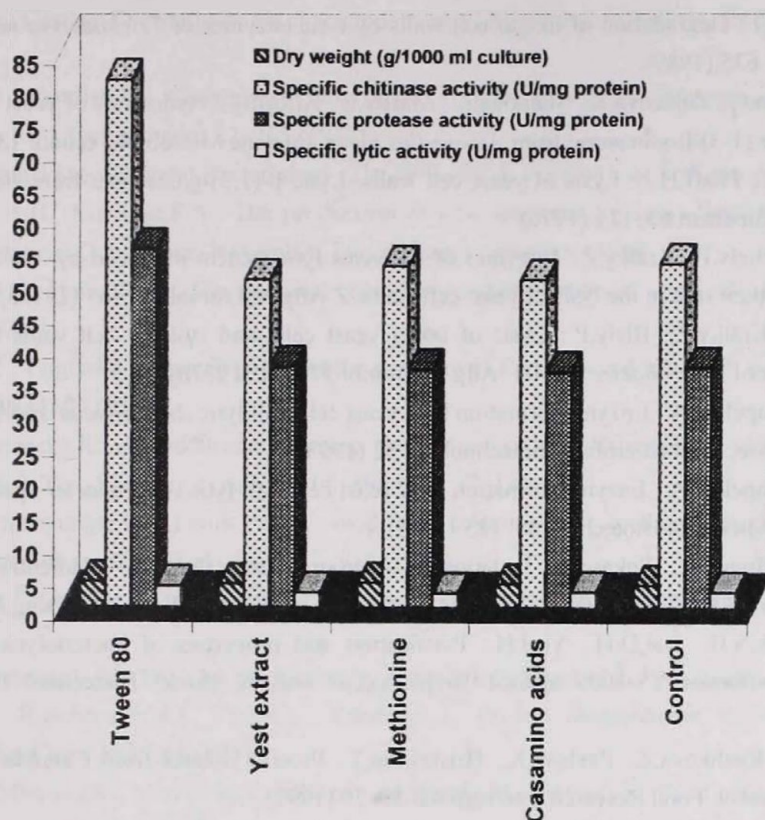


Fig. 8. Effect of Tween, yeast extract, methionine and casaminoacids on *Candida albicans* cell-wall lytic enzyme production and biomass yield of *S. thermodiastaticus*

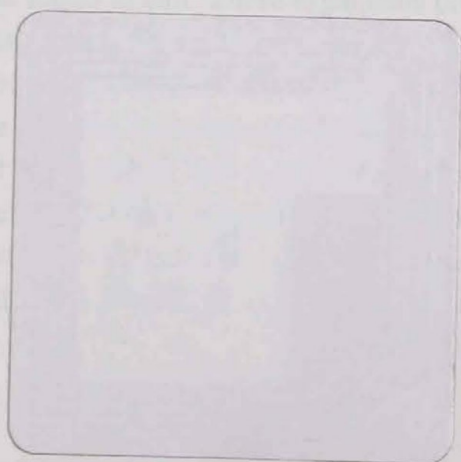
In general, it can be concluded that the production of the enzymes of the yeast cell-wall lytic system by *S. thermodiastaticus* was greatly influenced by the cultural conditions; the highest lytic activity was obtained when the strain was grown in a medium consisting of 1% *C. albicans* cell-wall, 0.1% NaNO_3 , 0.1% KH_2PO_4 , 0.0001% ZnSO_4 and 0.1% Tween 80, the pH of the medium was adjusted to 5.5 and the cultures were incubated at 50 °C for 18 h.

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PRODUCTION OF THAXTOMIN A BY TWO SPECIES OF *STREPTOMYCES* CAUSING POTATO SCAB

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A total of nine isolates of streptomycetes were isolated from scab lesions on potato tubers. Five out of them were pathogenic on potato minitubers and four of the pathogenic isolates produced thaxtomin A in infected tubers tissues. The lesion surface areas induced by thaxtomin A were highest in treatment of the minitubers with extract of OMB inoculated with S-6 and S-7, intermediate with that inoculated with S-4 and lowest with S-3. The pathogenic isolates were identified by their colour of aerial mycelia, melanin pigment productivity (+ or –), the type of spore chains morphology and carbon utilization as either *S. scabies* strains S-3, S-4 and S-8, or *S. acidiscabies* strains S-6 and S-7. S-3 and S-4 produced 0.65 and 1.60 µg thaxtomin A per milliliter of OMB, respectively, whereas S-6 and S-7 produced similar amounts of thaxtomin A, 2.36 and 2.10 µg per ml of OMB, respectively. The optimal temperature for production of thaxtomin A by *S. scabies* and *S. acidiscabies* was 28 °C. Production of thaxtomin A by *S. scabies* strain S-4 and *S. acidiscabies* strain S-6 was suppressed at least 50-fold at 0.5 and 0.3% of glucose, respectively. Fructose enhanced the production of thaxtomin A by both *S. scabies* and *S. acidiscabies*.

Keywords: thaxtomin A, *Streptomyces* sp., potato scab

Streptomyces species are a group of filamentous Gram-positive saprophytic bacteria found in soil. These organisms produce a great variety of antibiotics and other secondary metabolites with biological activity. A large proportion of these secondary metabolites are derived from amino acids [1, 2]. Many of secondary metabolites include phenolic or macrolide derivatives produced by a sequence of reactions similar to that responsible for fatty acids biosynthesis [3]. The chemistry, biosynthesis, molecular genetics and regulation of secondary metabolic compounds production have been studied extensively [4–6]. The secondary metabolites, including antibiotics production by *Streptomyces* are often associated with a low growth rate [5].

A few species of *Streptomyces* are plant pathogenic. *Streptomyces scabies* [7] is pathogenic to potato (*Solanum tuberosum* L.), causing an economically important disease of potato tuber called potato scab [8–10]. Common scab of potato occurs in all potato-growing areas of the world and may be induced by other species of *Streptomyces* as well [11–13]. *S. scabies* produces thaxtomins, phytotoxins, which are a series of unique 4-nitroindol-3-yl-containing 2,5-dioxopiperazines [14–17]. These toxins constitute a family of phytotoxic secondary metabolites that were isolated from *S. scabies*-infected plant tissue [16, 18], but are also produced in oatmeal based media [19–21]. Thaxtomins production in potato tuber tissues (were) correlated with pathogenicity on potato tubers by 23 strains of *S. scabies* [15], and may be quantitatively related to virulence within the same species [20]. In addition to *S. scabies*, thaxtomins are produced in plant tissue by two other pathogenic species, *S. acidiscabies* [22], the causal agent of acid scab on potato [20, 23], and *S. ipomoeae*, the cause of sweet potato soil rot and pox [24, 25].

The objectives of this study were to evaluate the pathogenicity of several isolates of *Streptomyces* species on potato minitubers and study the synthesis of phytotoxins associated with potato scab disease. In addition, examinations of the relationship between the taxonomy of pathogens and phytotoxin production, as well as on the regulation of toxin production by glucose and fructose are reported.

Materials and methods

Isolation and media

The method of Elesawy and Szabo [26] was used to isolate strains of streptomycetes from potato scab lesions with the following modifications. The scabbed tubers of potato were collected from different potato production areas in Sharkia governorate. The scab lesions were cut as one disk (1 cm diameter) from a randomly selected area on the surface of each 10 tubers to form composite sample. The composite sample (10 disks) was mechanically homogenized and diluted. Aliquots (0.5 to 1.0 ml) of the 10^{-2} and 10^{-3} dilutions were plated on glycerol-asparagine agar [27]. Five plates were prepared for each dilution, and all plates were incubated at 28–30 °C. After 10 days of growth, colonies were counted and maintained on slants of yeast-malt extract agar (ISP₂) [27].

Oatmeal broth (OMB) medium [20] amended with trace elements [19] was used for production of thaxtomin A. This medium was prepared with different concentrations of glucose or fructose at 0.0, 0.1, 0.3, 0.5 and 1.0% (w/v). OMB media

were inoculated with approximately 10^6 spore suspensions per flask. The cultures were incubated at 28 °C on a rotary shaker (150 rev/min) to obtain good aeration for 3–4 days in 500-ml Erlenmeyer flask. The cultures were filtered and the mycelial dry masses were determined.

Extraction and purification of thaxtomin A

The methods described by King et al. [16] and modified by Loria et al. [20] were used for extraction and purification of thaxtomin A. A standard thaxtomin A was generously provided by Russell R. King (Fredericton, Canada). Filtered liquid cultures (200 ml) were extracted twice with chloroform (100 ml). The crude extracts that remained after evaporation of chloroform to dryness were dissolved in acetone for testing scab-inducing activity or in chloroform for phytotoxin assay and analysis by TLC. For TLC analysis, crude extracts were taken up in chloroform and loaded onto silica gel thin-layer chromatography (TLC) performed on Merck silica gel 60 F₂₄₅ plates, and run in chloroform and methanol (9:1). The yellow bands were eluted from the silica and rechromatographed with the same conditions for further purification. The yellow bands comigrating with the authentic thaxtomin A were again eluted, dissolved in chloroform and evaporated to dryness. The purified yellow powder was stored in a desiccator at 4 °C and used for quantification.

Isolation of thaxtomin A produced by S. scabies and S. acidiscabies

The OMB cultures of *S. scabies* strains (S-3 and S-4) and *S. acidiscabies* strains (S-6 and S-7) with increasing yellow tint at 28 °C were harvested after 4 days. Bright yellow colours were observed after extraction of the media with chloroform and produced different strong yellow bands at $R_f = 0.65$ on silica TLC plates, which comigrated with thaxtomin A standard. When these bands were eluted and rechromatographed on silica TLC plates for further purification, a single intense yellow band for each isolate was visible at $R_f = 0.50$ and again comigrated with thaxtomin A standard. The developed spots were viewed under long-wavelength (390 nm) ultraviolet light using Min UVIS, DUOUV source for TLC. For purposes of quantifying the effect of varying culture conditions on production of thaxtomin A by *Streptomyces* spp., the areas under the bands (390 nm, thaxtomin A) were integrated, and the concentrations of thaxtomin A were deduced by comparison to standard curve generated from solutions of characterized thaxtomin A.

Thaxtomins were quantified by ultraviolet and visible spectroscopic analyses performed with a Deuterium UV/vis 21D spectrophotometer using extinction coefficient as determined by King et al. [16]. A standard curve for thaxtomin A was

established with dilutions of a known quantity of the purified compound. Thaxtomins were eluted with methanol and monitored at A_{380} [19].

Pathogenicity test and scab lesion formation

The maintained isolates were tested to identify the proportion of these isolates that are pathogenic to potato minitubers. Inocula were produced by inoculating oatmeal broth (OMB) amended with trace element [19]. Immature potato tubers cultivar Draga, which were obtained from Agricultural Research Center (Giza, Egypt), were used as criterion for pathogenic activity, according to the methods modified by Bukhalid and Loria [6]. Potato tuber disc (PTD) assay [20] was used to confirm the presence of thaxtomin A in culture extracts. Phytotoxins were also extracted from cultures of the tested isolates of *Streptomyces* grown in OMB for testing scab lesion formation [19]. Phytotoxin preparations were applied to 6 mm sterile filter paper discs and air-dried. The sample discs were applied directly to the potato minitubers.

Taxonomic criteria of isolated streptomycetes

The maintained isolates were transferred to inorganic salts-starch agar (ISP₄) [27]. After 14 days of incubation at 30 °C, the aerial mycelium and the type of spore chains morphology were determined [7, 22].

The melanin pigment productivity (+ or –) on tyrosine containing medium [27] is a distinguishing characteristic for taxonomy of pathogenic *Streptomyces* spp. [28]. The selected isolates were inoculated on glycerol-asparagine agar containing 0.3% tyrosine and incubated for 4–5 day at 28 °C.

The methods of Shirling and Gottlieb [27] were used to test the isolates for utilization of carbon. The tested carbon sources included L-arabinose, rhamnose, raffinose, I-inositol, D-xylose, D-mannitol, sucrose and D-fructose; D-glucose and no carbon served as positive and negative controls, respectively. Mycelia were washed twice by centrifugation at 4,600 g and resuspended in sterile distilled water, 0.05 ml of the suspension was plated on carbon utilization media [29].

Results and discussion

Pathogenicity and lesion formation. A total of nine isolates of streptomycetes with light grey colonies were isolated from potato scab lesions and screened for pathogenicity on minitubers of the potato cultivar Draga. Only five isolates were pathogenic to potato minitubers (Table I). All isolates that were pathogenic except

isolate S-8 also displayed evidence for the involvement of the thaxtomin A. Thaxtomins were produced by *Streptomyces scabies*, *S. acidiscabies* and *S. ipomoeae* [16, 25], suggesting that this family of phytotoxins may be a pathogenicity determinant in *Streptomyces* species. The positive correlation between the pathogenicity of the four streptomyces isolates and their ability to produce thaxtomin A were in accordance with that obtained by Lawrence et al. [17], King et al. [15] and Slabbert et al. [18].

Table I

Pathogenicity test and thaxtomin A assay for Streptomyces isolates cultivated in OMB medium

<i>Streptomyces</i> isolates	Pathogenicity ^a on PMT	Extract effect	Thaxtomin A ^b in extract
S-1	—	—	—
S-2	—	—	—
S-3	+	+	+
S-4	+	+	+
S-5	—	—	—
S-6	+	+	+
S-7	+	+	+
S-8	+	—	—
S-9	—	—	—
Control	—	—	—
Chloroform	—	—	—

^aFive immature potato minitubers (PMT) were immersed in OMB cultures of the tested isolates for 5 min followed by incubation at relatively high humidity for 3–4 days at 22–25 °C in dark

^bThaxtomins were extracted from OMB cultures and applied to PTD. Extracts from uninoculated OMB and chloroform were used as control

+ = presence of thaxtomin A in PTD

— = absence of thaxtomin A in PTD

Cultures of OMB media were inoculated with the nine isolates tested and incubated at 28 °C for 3–4 days. Thaxtomins were extracted from the cultures and tested for their ability to induce lesion formation on potato minitubers (var. Draga). The results of lesion formation (Table II) demonstrate that the scab-inducing compounds were produced by four isolates (S-3, S-4, S-6 and S-7) only, and that toxins by S-6 and S-7 induced more severe scab symptoms on the susceptible potato cultivar Draga than did isolates S-3 and S-4 (Fig. 1). The lesion produced by scab-inducing toxin on potato

tubers ranged from browning (slightly raised) to erupted tuber's skin (deeply pitted). The production of thaxtomin A by these isolates, at different concentrations, may partially determine the lesion type, as probably other members of the thaxtomin family [25]. The expansion of lesion surface areas on potato minitubers were produced by thaxtomin A at concentrations of 10^{-5} to 10^{-6} , and superficial lesions may be produced when the tubers are exposed to low concentration of toxin [17]. Babcock et al. [19] reported that scab symptoms included browning and eruption of tuber's skin at the site of phytotoxin application. High concentrations of thaxtomin A at the infection site may cause cell death, which ultimately results in a pitted lesion. Loria et al. [20] found that more toxin and more necrosis were produced by *S. scabies* strain 87-22, which was isolated from a pitted lesion.

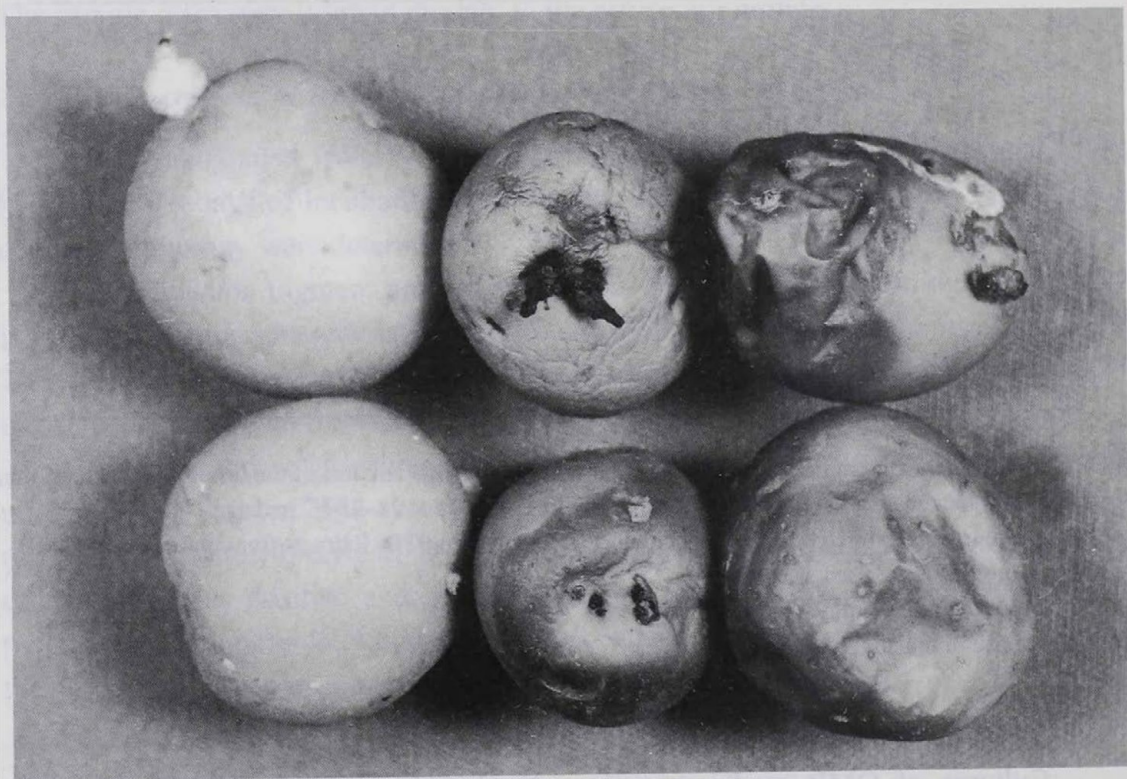


Fig. 1. Scab lesion formation on Draga minitubers. Potato minitubers (cv. Draga) were treated with (a) extract of uninoculated OMB, (b) acetone, or (c) phytotoxin extract from S-4, (d) from S-3, (e) from S-7 and (f) from S-6 grown in OMB. The darkened areas on the tubers (c), (d), (e) and (f) correspond to scab lesions.

Table II

Lesion formation on potato minitubers induced by thaxtomin A

<i>Streptomyces</i> isolates	Severity of lesion formation* (No. of minitubers)			
	–	+	++	+++
S-1	10	0	0	0
S-2	10	0	0	0
S-3	4	1	2	3
S-4	2	1	2	5
S-5	10	0	0	0
S-6	0	0	2	8
S-7	0	0	3	7
S-8	10	0	0	0
S-9	10	0	0	0
Control	10	0	0	0
Acetone	10	0	0	0

Thaxtomin A was extracted by acetone from different *Streptomyces* isolates grown on OMB and applied to minitubers of potato cultivar Draga. Extract from uninoculated OMB and acetone were used as control.

*Ten minitubers were used for each treatment and scored for severity of lesion formation as follows: – healthy tubers; + brown to dark skin; ++, blackened tissue; +++, blackened and erupted skin.

Taxonomy of the pathogenic isolates. The morphological and physiological properties of the pathogenic isolates of streptomycetes are shown in Table III. S-3, S-4 and S-8 were similar to *S. scabies* in taxonomic characteristics such as spiral chain sporophore, production of melanin pigment and pathogenicity to potato minitubers, and the isolates of S-6 and S-7 were similar to *S. acidiscabies* with flexuous spore chains morphology without melanin pigment production. All the pathogenic isolates tested had complete carbon-utilization patterns, but S-6 and S-7 did not utilize raffinose (data not shown). The use of ISP criteria, either by Shirling and Gottlieb [27] or modified by Lambert and Loria [7, 22] proposed the revival name *S. scabies* for those being characterized by smooth gray spores borne in spiral chains, as compared to another pathogenic species (*S. acidiscabies*) which has white flexuous spore chains morphology.

Table III

Production of thaxtomin A in relation to the taxonomy of pathogenic Streptomyces species

Strain	Spore chain morphology	Melanin production	Thaxtomin A production (µg/ml)
S-3	Spiral	Positive	0.65
S-4	Spiral	Positive	1.60
S-8	Spiral	Positive	ND
S-6	Rectiflexible	Negative	2.36
S-7	Rectiflexible	Negative	2.10

ND: not detected (less than 0.05 µg/ml)

Elesawy and Szabo [30] found that only one isolate of *S. scabies* had non-smooth spore surface indicating that spore surface is not a useful criterion in distinguishing between *S. scabies* and other pathogenic species of streptomycetes that are morphologically and physiologically different [28]. The identification method of Williams et al. [31], involving colours of spores, colony underside, any pigment produced in the medium and production of melanin was not sensitive enough. This method modified by excluding the colours of the colony underside, which is not distinctive [32] for several *Streptomyces* species inducing potato scab, and pigment other than melanoid pigment (none is produced), and substituting criteria based on a combination of spore chain morphology (SI or RF) and melanin pigment productivity (+ or –) were included by Tashiro et al. [33] and Tanaka [28].

Thaxtomin A production was quantitatively determined in the five isolates that had distinct pathogenic activity to potato. The results presented in Table III indicate that all isolates, with one exception (S-8), produced thaxtomin A, and the rectiflexible (RF) strains produced phytotoxin more than did spiral strains. The identification of strains S-3, S-4 and S-8 as *S. scabies*, and S-6 and S-7 as *S. acidiscabies*, coincided with the pattern of toxin production of pathogenic strains of *Streptomyces*. These results agree with those of RF type strains of *S. acidiscabies* tending to produce more thaxtomin A than did the spiral strains published by Loria et al. [20] and Natsume et al. [23].

The lack of thaxtomin A production by S-8, which has pathogenic activity, supports the findings of Tashiro et al. [33] and Natsume et al. [23] who suggested that the loss of ability of a pathogenic strain, resembling *S. scabies* (S-131), to produce thaxtomin A is due to prolonged preservation and/or repeated subculturing.

Regulation of thaxtomin A production. OMB media were inoculated with *S. scabies* (S-4) and *S. acidiscabies* (S-6). These two strains were chosen in regulation studies of thaxtomin A production since they had the highest phytotoxin production. The two strains were incubated at 25, 26, 28, 30 and 32 °C. After 4 days of incubation cell filtrates were extracted and amounts of thaxtomin A were measured. Table IV shows that 28 °C was the optimal temperature for thaxtomin A production. Thaxtomin A production at 28 °C by S-4 was 25.0, 16.8, 12.5 and 38.7% higher than production at 25, 26, 30 and 32 °C, respectively. Thaxtomin A production by S-6 was significantly reduced by 23.7, 15.2, 10 and 33% at 25, 26, 30 and 32 °C compared with production at 28 °C, respectively. On the other hand, S-4 and S-6 did not grow well at temperatures below 25 °C or above 32 °C. These results were in accordance with those obtained by Babcock et al. [19].

Table IV

Effect of temperature on production of thaxtomin A and on mycelial dry mass of S. scabies and S. acidiscabies (% = reduction of thaxtomin A)

Temperature, °C	Thaxtomin A production (µg/ml)				Mycelial dry mass (g/100 ml)	
	S-4	%	S-6	%	S-4	S-6
25	1.19	25.0	1.80	23.7	0.04	0.025
26	1.33	16.8	2.00	15.2	0.09	0.065
28	1.60	0.0	2.36	0.0	0.12	0.085
30	1.40	12.5	2.12	10.0	0.13	0.11
32	0.98	38.7	1.58	33.0	0.08	0.07
LSD _{0.01}	0.62		0.20		0.040	0.027
LSD _{0.05}	0.45		0.12		0.025	0.018

S. scabies (S-4) and *S. acidiscabies* (S-6) were grown at 28 °C on a rotary shaker (150 rev/min) in OMB medium supplemented with 0, 0.1, 0.3, 0.5 and 1.0% of glucose (w/v), and the amount of thaxtomin A in each extract was quantified by spectrophotometer at A₃₈₀. The data presented in Table V indicate that *S. scabies* and *S. acidiscabies* produced 1.60 and 2.36 µg thaxtomin A per milliliter of OMB, respectively. By adding to OMB cultures of *S. scabies* 0.1, 0.3, 0.5 and 1.0% of glucose, thaxtomin A was significantly reduced by 12.5, 26.2, 53.1 and 66.2%, respectively, and 19.5, 51.7, 65.2 and 71.2% reduction of the thaxtomin A was produced by *S. acidiscabies* at the same concentrations, respectively. In contrast,

growth of the two tested *Streptomyces* species increased with an increasing concentration of glucose, and growth did not correlate to thaxtomin A production. *S. scabies* was recently reported to produce thaxtomin A in OMB, and the production was suppressed by glucose in this species [19, 20]. Suppression of many of the secondary metabolites by glucose is also common among streptomycetes [5, 34].

Table V

Thaxtomin A production and mycelial dry mass of S. scabies and S. acidiscabies in OMB medium supplemented with different concentrations of glucose (% = reduction of thaxtomin A)

Glucose concn (%)	Thaxtomin A production (µg/ml)				Mycelial dry mass (g/100 ml)	
	S-4	%	S-6	%	S-4	S-6
0.0	1.60	0.0	2.36	0.0	0.13	0.11
0.1	1.40	12.5	1.90	19.5	0.17	0.20
0.3	1.18	26.2	1.14	51.7	0.22	0.25
0.5	0.75	53.1	0.82	65.2	0.38	0.31
1.0	0.54	66.2	0.68	71.2	0.60	0.43
LSD _{0.01}	0.83		0.31		0.055	0.020
LSD _{0.05}	0.48		0.14		0.039	0.011

Table VI

Thaxtomin A production and mycelial dry mass of S. scabies and S. acidiscabies in presence of different concentrations of fructose in OMB medium (% = stimulation of thaxtomin A)

Fructose concn (%)	Thaxtomin A production (µg/ml)				Mycelial dry mass (g/100 ml)	
	S-4	%	S-6	%	S-4	S-6
0.0	1.60	0.0	2.36	0.0	0.13	0.11
0.1	1.84	15.0	2.50	5.9	0.15	0.13
0.3	2.16	35.0	3.17	34.3	0.21	0.20
0.5	2.55	59.3	3.60	52.5	0.27	0.32
1.0	2.94	83.7	4.25	80.0	0.45	0.35
LSD _{0.01}	0.39		0.43		1.021	0.063
LSD _{0.05}	0.24		0.14		0.018	0.035

Fructose as a component of OMB supported thaxtomin A production by *S. scabies* and *S. acidiscabies* strains. Various fructose concentrations (0.0, 0.1, 0.3, 0.5 or 1.0%) were added to OMB medium to assess their effect on thaxtomin A biosynthesis and dry mass. Fructose concentrations were found to significantly enhance biosynthesis of thaxtomin A and increase mycelial dry mass of the two tested species (Table VI). Production of thaxtomin A by *S. scabies* at 0.1, 0.3, 0.5 or 1.0% of fructose was 15, 35, 59.3 or 83.7% higher than at 0.0 concentration, respectively. On the other hand, thaxtomin A production by *S. acidiscabies* was stimulated by 5.9, 34.3, 52.5 or 80% at 0.1, 0.3, 0.5 or 1.0% of fructose compared with the control, respectively. The mycelial dry weight of *S. scabies* was 0.13, 0.15, 0.21, 0.27 and 0.45 g, and of *S. acidiscabies* 0.11, 0.13, 0.20, 0.32 and 0.35 g per 100 ml of OMB supplemented with 0.1, 0.3, 0.5, or 1.0% of fructose, respectively.

Interestingly, Vilches et al. [35] reported that only about 20% of fructose was consumed by *S. antibioticus* at the end of growth period, and the slow fructose utilization was not due to the lack of an uptake system for this sugar. Instead, such an uptake system (which is present in this organism) requires some time for induction of oleandomycin. Loria et al. [20] suggested that the high concentration of fructose in OMB may stimulate growth and thaxtomin A production, and Quigley and Gross [36] demonstrated that production of phytotoxin syringomycin by *Pseudomonas syringae* pv. *syringae* was enhanced by fructose.

The present study has demonstrated that pathogenic isolates of *Streptomyces* spp. can be recovered from tuber surfaces and from their rhizosphere after disease development. The limited information available about the pathogenic *Streptomyces* spp. is probably due to the lack of selective isolation techniques. The production of thaxtomin A by *S. scabies* and *S. acidiscabies* was considered to be the dominant toxic compound, and the regulation of its biosynthesis is similar to that of other secondary metabolites produced by streptomycetes. The ability to produce thaxtomin A in large quantities provides the means for using this compound as a screen in testing potato cultivars for resistance to scab disease.

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CIGARETTE SMOKE DECREASES THE EXPRESSION OF SECRETORY COMPONENT IN HUMAN BRONCHIAL EPITHELIAL CELLS, *IN VITRO*

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Epithelial secretory component (SC) is thought to be essential for immunologic protection of the respiratory tract from viral and bacterial infection, since it transports polymeric IgA from the basolateral to the luminal surface of epithelial cells. We have hypothesized that recurrent infection in airways of cigarette smokers is at least partly a consequence of cigarette smoke-induced downregulation of the expression and/or release of SC from airway epithelial cells, subsequently resulting in decreased transcytosis of secretory IgA to the airway lumen.

To test this hypothesis, we have cultured human bronchial epithelial cells (HBEC) from surgical tissues and exposed these for 20 minutes to either air or cigarette smoke. Following exposure to cigarette smoke the HBEC cultures were incubated for a further period of up to 24 h, during which time separate cultures were processed by immunocytochemistry for the presence of SC, in a time-dependent manner. The stained HBEC cultures were evaluated by colour image analysis for the percentage of total cells staining for SC.

Exposure to cigarette smoke significantly decreased the percentage of total HBEC staining for secretory component from a baseline value (median and interquartile[IQ]1, IQ3) of 35.9% (26.5, 41.6) to 15.7% (8.2, 25.4; $p < 0.05$) 1 h after exposure, compared with exposure to air. The percentage of cells staining for secretory component were further reduced to 5.3% (3.3, 6.4; $p < 0.01$), 6 h after exposure, compared to exposure to air. After incubation for 24 h following exposure to cigarette smoke, there was gross cell damage and the cells were not suitable for immunocytochemical analysis.

These results suggest that short-term exposure to cigarette smoke may compromise the immune barrier function of the airway mucosa by decreasing the expression and/or

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release of epithelial SC, thereby decreasing the transcytosis of IgA necessary for inactivating the microbial pathogens in the airway lumen.

Keywords: human bronchial epithelial cells, secretory component, cigarette smoke

Several studies have demonstrated that the frequency of respiratory infections and severity of symptoms are increased in both patients with chronic respiratory disease and healthy smokers. Some studies have suggested that there is a link between exposure to cigarette smoke and incidence of respiratory tract infection [1, 2]. Indeed, Kershaw [3] has demonstrated that passive smoking significantly increased wheezing, coughing and respiratory infections in children of mothers who smoked, and that this effect was directly proportional to the number of cigarettes smoked by the mother. It has been postulated that the increased respiratory infection in cigarette smokers is a consequence of cigarette smoke-induced changes in mechanisms of the host defense system, including impairment of ciliary function, increase in the volume of mucus, changes in the humoral response to antigens, and quantitative and qualitative changes in cellular components.

More recent evidence suggests that changes in the expression of secretory component (SC), a glycoprotein expressed basolaterally in epithelial cells of a variety of tissues, including airway epithelial cells [4, 5], may play a vital role in the protection of the respiratory tract from bacteria and viruses. Several studies have demonstrated that SC acts as a polymeric immunoglobulin receptor (pIgR) and mediates the transcytosis of locally produced polymeric immunoglobulin (pIgA and pIgM) to the luminal surface of the cells [4]. Studies of human intestinal epithelial cell lines have demonstrated that treatment with several cytokines, including interleukin(IL)-1 α , IL-1 β , IL-4, tumour necrosis factor(TNF)- α and interferon(IFN)- γ , leads to enhanced membrane expression and secretion of SC from these cells [5–7]. In contrast, Gregory and Gfell [8] have demonstrated that culture of these cells in the presence of different concentrations of either an aqueous extract of smokeless tobacco or pure nicotine significantly decreased the amounts of SC released into the culture medium, compared with untreated cultures. Furthermore, these authors demonstrated that the tobacco-induced decrease in SC release could be attenuated by the addition of IL-4 or IFN- γ to the cultures. In view of these findings, we hypothesised that recurrent infection in the airways of cigarette smokers is, at least in part, a consequence of decreased transcytosis of secretory IgA to the airway lumen, resulting from cigarette smoke-induced down-regulation of airway epithelial secretory component. In order to test this hypothesis we cultured human bronchial epithelial cells, from surgical tissues, and exposed these for

20 minutes to either air or cigarette smoke, followed by immunocytochemical processing and evaluation of the cultures for the presence of secretory component.

Materials and methods

All chemicals and reagents were of tissue culture grade and unless stated otherwise, were obtained from the Sigma Chemical Co (Poole, England).

Culture of human bronchial epithelial cells (HBEC)

Bronchial tissue was obtained from four males and two female patients, who underwent lobectomy for lung cancer at The London Chest Hospital, London, UK. All patients were smokers, not allergic to common allergens, and of mean age = 67.9 ± 4.2 years (range 60–79 years). Following resection, only tissue macroscopically free of tumour was processed further for culture.

HBEC were cultured using the explant cell culture technique developed in our laboratory [9]. Briefly, the epithelium was dissected away from the underlying lamina propria and after further dissection into smaller sections of approximately 0.5 mm^3 , the epithelium was washed three times with fresh sterile Medium 199. 2–3 pieces of the washed epithelium were explanted onto washed and sterilised glass microscope slides, and then incubated at 37°C in a 5% CO_2 in air atmosphere in 90 mm diameter Falcon Petri dishes (Becton Dickinson Ltd, Oxford, England), each containing 7.0 ml of culture medium. The culture medium was prepared by supplementing medium 199 with 2.5% foetal calf serum and a variety of growth factors, including human transferrin ($2.5 \text{ }\mu\text{g/ml}$), epidermal growth factor (20 ng/ml), bovine pancreatic insulin ($2.5 \text{ }\mu\text{g/ml}$), hydrocortisone ($0.36 \text{ }\mu\text{g/ml}$), L-glutamine (0.02 mg/ml) and 1.5% (v/v) antibiotic and antimycotic solution (composed of penicillin, streptomycin and amphotericin B). After incubation for three days, the medium in the culture dish was replaced with fresh medium supplemented with 2.5% NUSERUMTM IV Culture Supplement (Universal Biologicals Ltd, London, England) and the cultures were observed for epithelial cell outgrowth. The culture medium was replaced every 48 hours until the outgrowing cells had grown to confluence, normally by 3–4 weeks.

The identity of the epithelial cells was confirmed in all cultures by light microscopy and in randomly selected cultures by electron microscopy and immunocytochemical staining for cytokeratin using monoclonal antibody preparation CAM 5.2 (Becton Ltd, Oxford, England, UK). Randomly selected cultures were also assessed for any contaminating T-lymphocytes, B lymphocytes, fibroblasts, mast cells, neutrophils and macrophages, using monoclonal antibody preparations CD3, CD37

(Serotec Ltd, Oxford, England, UK), 5B5, AA1, NP57 and Ber-MAC3 (Dako Ltd, (Bucks, England), respectively.

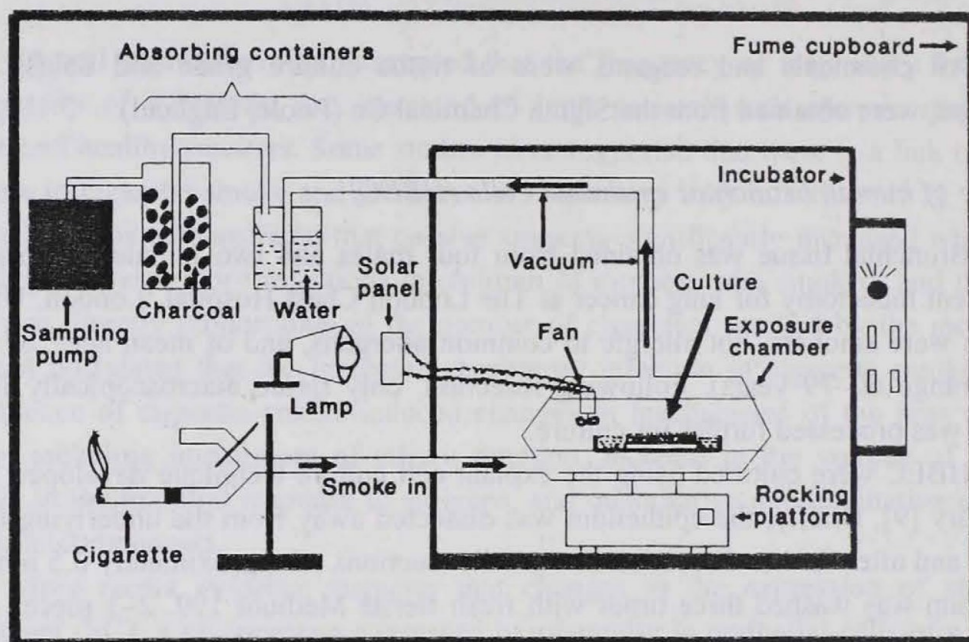


Fig. 1. Exposure system of cultures to cigarette smoke

Exposure of HBEC cultures to cigarette smoke

Exposure of HBEC cultures to cigarette smoke was carried out in an exposure system developed in our laboratory (Figure 1). Cigarette smoke was generated by burning commercially available Superkings cigarettes (12 mg tar, 1.1 mg nicotine) and drawn through a 5.5 litres volume airtight polycarbonate exposure chamber (Billups Rothenberg, Del Mar, CA, USA). The flow rate of the cigarette smoke was adjusted to 850 ml/min by use of a variable flow (5–3000 ml/min) Airchek 50 Sampling Pump (SKC Ltd, Dorset, England), connected to the top of the exposure chamber. The cigarette smoke was evenly dispersed within the exposure chamber by use of a small solar energy powered fan, deriving energy from an illuminated 100 W light bulb, which was placed just below the HBEC cultures inside the chamber. Under these conditions, each cigarette burned for five minutes, on average.

To facilitate direct exposure of HBEC to cigarette smoke, the exposure chamber was rocked gently, at intervals of 2.5 seconds, to an angle of 10° from the horizontal in

each quarter of the horizontal plane on a Luckham 4RT rocking table (Luckham Ltd, Burgess Hill, England), thereby momentarily displacing approximately half the medium covering the surface of the culture plate during each tilt. To ensure to keep the temperature stable at 37 °C during exposure, the entire system was placed in a 60 litre capacity SI.60 incubator (Stuart Scientific, Redhill, England) made of acrylic material.

HBEC cultures were exposed to cigarette smoke for 20 minutes and then transferred to a tissue culture incubator at 37 °C in a 5% CO₂ in air atmosphere, for up to 24 hours. Over the course of this period, incubation was terminated at times 1, 3, 6 and 24 hours for different sets of HBEC cultures, and all cultures were air dried prior to processing by immunocytochemistry and colour image analysis for evaluation of HBEC expressing SC. Appropriate controls were also prepared by exposing HBEC cultures to air for 20 minutes and then treating further as for the test cultures.

Immunostaining of HBEC cultures for SC

HBEC cultures were stained for secretory component, using a GA-1 mouse anti-human secretory component antibody (Cat No: I6635), according to a standard protocol.

All air dried HBEC cultures were fixed in fresh acetone for 5 min at room temperature and following air drying for 10 min, were washed in PBS for 5 min. The cultures were then incubated in 0.5% hydrogen peroxide in methanol for 15 min to block the activity of any endogenous peroxidase, and washed further for 5 min in distilled water and PBS. Any remaining endogenous peroxidase was blocked by adding 1% normal horse serum (Dako Ltd, Cambridge, UK) for 15 min, and following a PBS wash the HBEC cultures were incubated with 1:200 dilution of anti-Human Secretory Component antibody for 2.5 hours. At the end of this incubation the HBEC cultures were washed again in PBS for 5 min and then incubated with 1:50 dilution of biotinylated rabbit anti-mouse antibody containing 1% normal swine serum (Dako Ltd, Cambridge, UK) for 45 min. The HBEC were then washed in PBS for 5 min and incubated further with Avidin-biotin complex for 30 min, and then with fresh filtered DAB for 5-10 minutes. The HBEC were rinsed with distilled water for 10 min, and then counterstained with Mayers hematoxylin for 5-10 min. Following a final rinse in tap water, the stained HBEC were dehydrated through a series of ascending concentrations of alcohol, cleared in xylene and finally mounted in DPX.

Quantitation of SC in HBEC cultures

All cultures stained for SC were coded and then observed and analyzed by an individual blinded to the treatment regimes. Expression of SC in HBEC cultures was quantified by means of a "FREELANCE" computerised colour image analysis and

processing system (Sight System, Hove, England), according to a modification of the technique described by Poston and colleagues [10]. Briefly, each culture was viewed through an Olympus BH2 microscope, at $\times 40$ magnification, and the viewed image transported onto a high intensity colour monitor, via a JVC TH-1070E colour video camera. The colour produced by the peroxidase reaction within the HBEC cultures was detected and measured as pixels of hue, saturation and intensity. Each HBEC culture was assessed by viewing 20 adjacent high magnification fields and the results were expressed as the percentage of HBEC in each field staining for SC. Colour image analysis was calibrated against the negative control before measuring the positive staining for secretory component in each culture.

Statistical analysis

Data were tested for normality using a normal probability plot and the Shapiro-Wilk test, and then analyzed further using non-parametric analysis of variance and Mann Whitney U test.

Results

Culture and identification of HBEC

Microscopic examination of HBEC using Hoffman modulation contrast optics demonstrated large numbers of cells with polygonal morphology, typical of epithelial cells, growing out from the explants, which reached confluence normally after three weeks. Indirect immunoperoxidase staining with CAM 5.2, revealed that all the cells in the cultures stained for cytokeratin confirming the epithelial nature of the cells. Staining with specific monoclonal antibodies, as listed in the Methods section against contaminating cell types including endothelial cells, fibroblasts, muscle cells, macrophages, T and B lymphocytes, mast cells, eosinophils and neutrophils did not show any indication of these cells.

Staining for secretory component

Light microscopic examination of the HBEC cultures demonstrated that these were not stained homogeneously for constitutively expressed SC, but rather that staining was very marked in dispersed groups of cells (Figure 2). In contrast, controls stained with an irrelevant antibody, instead of anti-SC antibody, showed no staining.

Estimation, by colour image analysis, of the percentage of SC-staining HBEC, demonstrated that 35.9% (26.5, 41.6; median and interquartile range) of HBEC

constitutively expressed SC. Exposure for 20 minutes to cigarette smoke significantly decreased the percentage of SC-staining HBEC to 15.7% (8.2, 25.4; $p < 0.05$), 1 h after exposure, compared with HBEC exposed to air for 20 minutes. The percentage of cells staining for SC were further reduced to 6.3% (4.1, 10.3; $p < 0.01$) and 5.3% (3.3, 6.4; $p < 0.01$), 3 h and 6 h after exposure, respectively, compared to exposure for 20 minutes to air (Figure 3a, b, c and Figure 4). At 24 h following exposure to cigarette smoke, there was gross cell damage and loss of integrity of the cultures, making these unsuitable for further investigation by immunocytochemistry (Figure 3d and Figure 4).

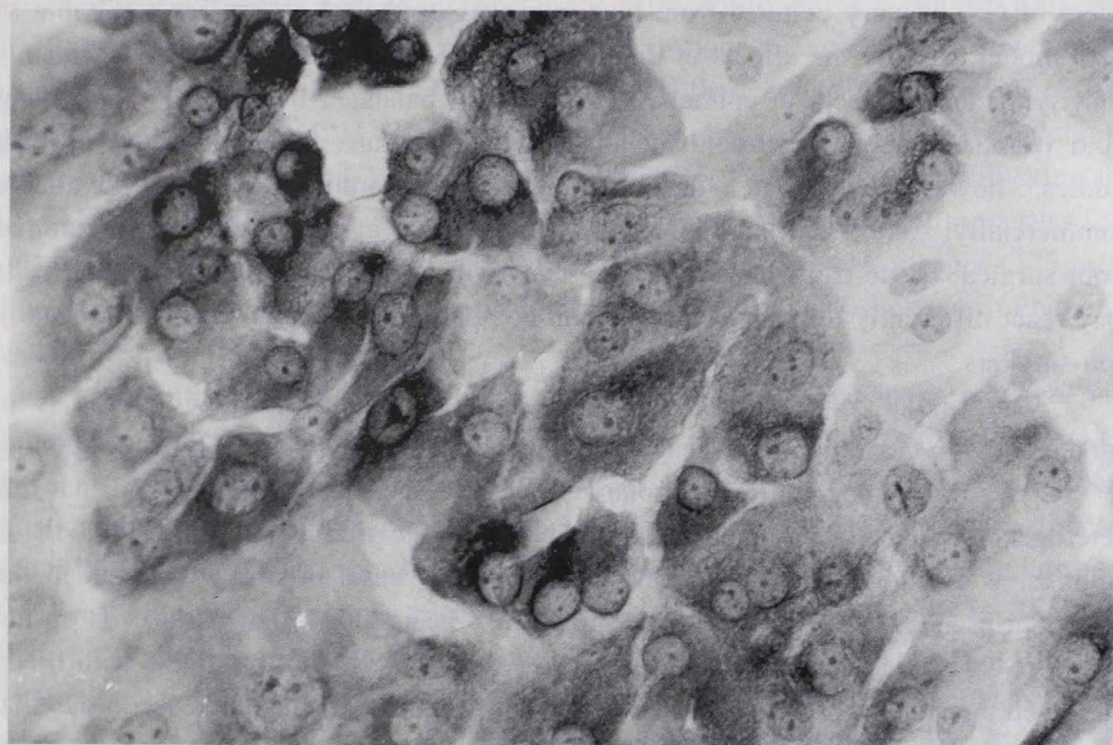


Fig. 2. Three-week-old human bronchial epithelial cell culture stained for secretory component

Discussion

In this study we have demonstrated that 20 minutes exposure of HBEC cultures to cigarette smoke significantly reduced the expression of SC in these cells. To our knowledge this is the first report on the effect of cigarette smoke on SC under experimental conditions.

We have previously demonstrated that HBECs cultured to confluence *in vitro* retain morphological and biochemical characteristics similar to those found *in vivo* [11, 12], and therefore render this model useful in the study of epithelial cell physiology. Characterization of the different epithelial phenotypes that constitute the HBEC cultures demonstrated that of the total, approximately 0.5% are goblet cells, 40–45% are ciliated epithelial cells, 35–40% are glandular epithelial cells and 20% are basal epithelial cells [12].

Despite kinetic limitations and the lack of naturally occurring defence mechanisms such as the presence of a “protective” epithelial lining fluid and endogenous “reducing” agents which may limit the oxidant-induced cell damaging effect of CS, primary cultures from human airway epithelial cells offer a suitable *in vitro* model to study the effects of direct exposure to this agent and the mechanism/s underlying these effects in epithelial cells. A well balanced human epithelial lining fluid would undoubtedly present the most optimal culture medium for use in such studies, however, to date such standardised preparations are not available commercially. A further limitation of this study is that epithelial cells were cultured from surgical tissue from lung cancer patients with a history of smoking: such cells may react differently to oxidant stress when compared to epithelial cells from healthy non-smokers.

The finding of non-homogenous staining for SC in HBEC cultures is in agreement with Godding and colleagues [5], who found that without IFN- γ , the staining for SC was not homogenous in unpolarized HBEC. These authors suggested that this heterogeneity in cellular content and/or heterogenous production of SC by HBEC cultures, is possibly related to their original *in vivo* basal or apical location in the bronchial pseudostratified epithelium, since it has been shown that basal cells do not express SC [13–15]. They also showed, however, that incubation of HBEC with IFN- γ increased the amount of SC produced by the cells, together with an increase in the total number of cells producing SC.

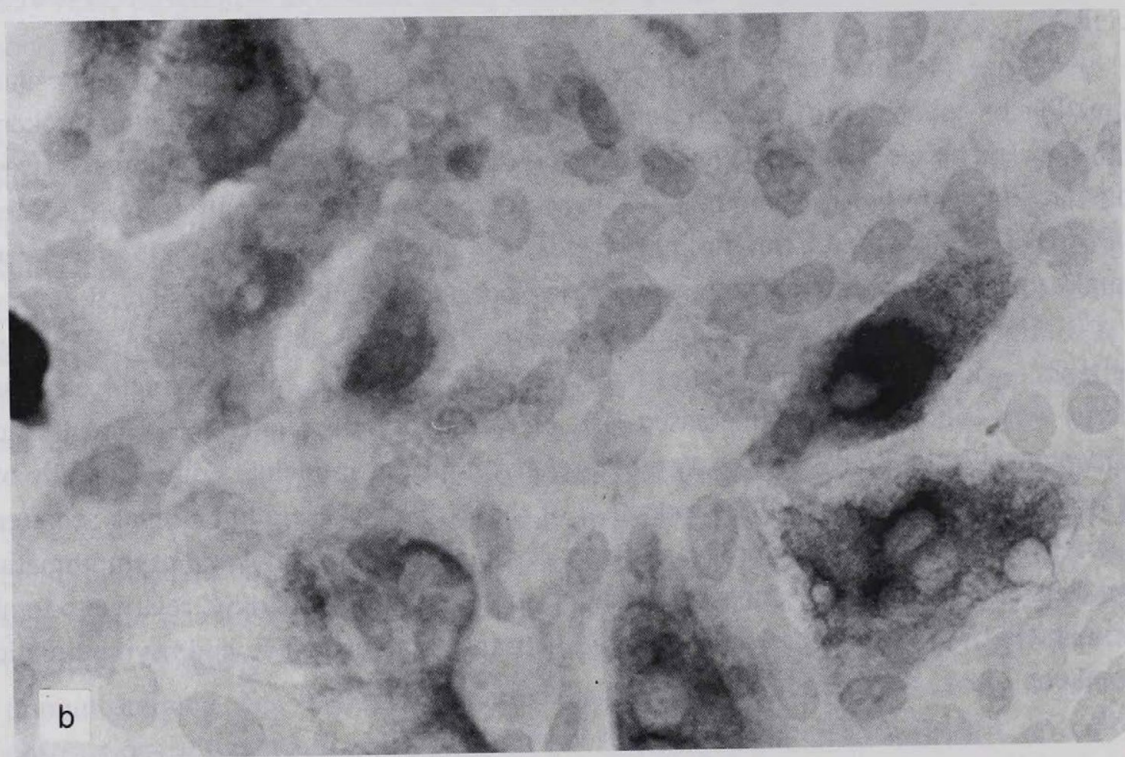
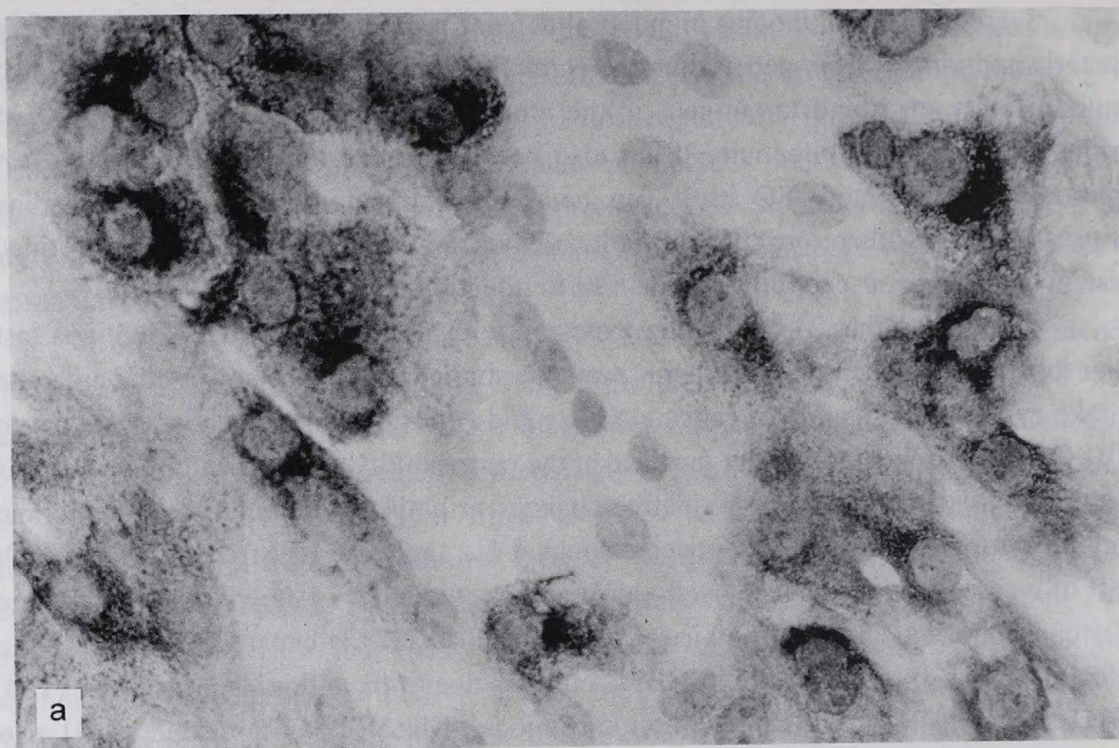
Decreased SC level has been associated with various pathologies. Diminished SC levels were described in the bronchoalveolar lavage fluid of asthmatics compared to healthy normal controls [16], whilst transient salivary IgA deficiency during the first year of life was linked to the later development of bronchial hyperreactivity [17]. Most recently, Godding and colleagues [18] reported that the intensity of specific immunostaining for SC in the bronchial epithelium correlates with bronchial obstruction in patients with chronic obstructive pulmonary diseases (COPD), suggesting that impaired bronchial epithelial secretory function may parallel airway obstruction in COPD.

Secretory IgA antibodies in mucosal defense provide an immune barrier to keep bacteria and viruses from penetrating the epithelium, thereby preventing infection. IgA antibodies can also bind to antigens in the mucosal lamina propria and remove them through the adjacent epithelium. It has also been suggested that even before the IgA antibodies are secreted, SC itself can neutralize intracellular pathogens including viruses [19]. It is, therefore, not difficult to envisage that perturbation of this refined system can lead to susceptibility of the host to infection.

Our finding that 24 hours after exposure to cigarette smoke HBEC cultures lost their integrity is in agreement with our previous studies where we showed that cigarette smoke exposure is capable of causing a dose and exposure time dependent damage to HBEC cultures together with an increase in the permeability of these cultures [20]. It is likely that this was caused by oxidative changes in both the epithelial cell membrane and the cytoskeleton, since it has been estimated that there are 10^{14} free radicals in each puff of cigarette smoke [21]. Indeed, our previous studies have shown, that glutathione at a concentration found in the airway lining fluid *in vivo* is capable of blocking the effect of CS or ozone exposure in HBEC cultures due to its antioxidant properties [20, 22]. Using a different epithelial cell culture model, MacNee and colleagues [23] also found that oxidant stress resulted in profound changes in the cytoskeleton of epithelial cells.

Since the lung is the first target organ which is affected in its structure and function by inhalation of cigarette smoke, it is not surprising that cigarette smokers have an increased risk of developing recurrent respiratory tract infections, airway obstruction, and clinical symptoms leading to chronic obstructive pulmonary diseases (COPD) [24]. In children, on the other hand, even passive cigarette smoking has been implicated in upper respiratory tract infections, otitis media and bronchitis [1, 2].

Our finding that cigarette smoke exposure decreases the expression of SC in HBECs, which is the cornerstone of the mucosal immunity, might explain at least partly why smokers are at increased risk of respiratory tract infection. Merrill and colleagues [25] hypothesised, that smoke-related injury to bronchial epithelial cells of smokers is reflected in the SC production of these cells. These authors analysed free secretory component (FSC) in lavage fluids obtained from nonsmokers, asymptomatic smokers and symptomatic smokers. Among symptomatic smokers, FSC relative to total protein (FSC/TP) was depressed compared with that in nonsmokers and asymptomatic smokers.



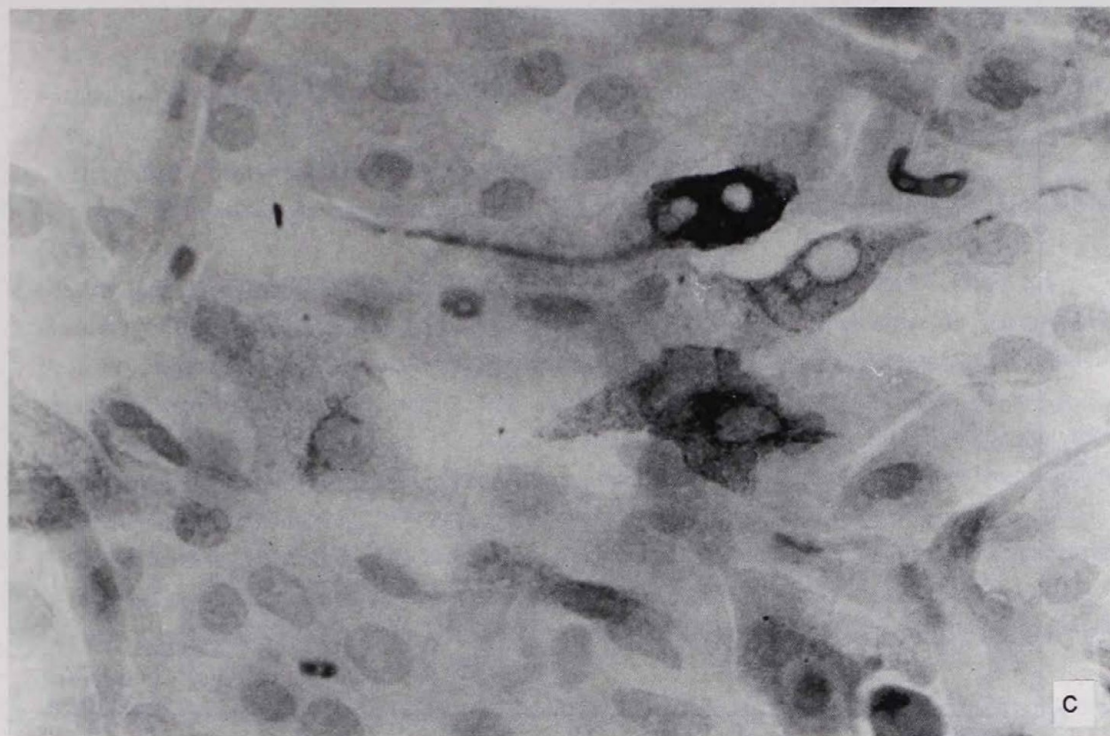


Fig. 3. The effect of 20 minutes exposure to cigarette smoke on the expression of secretory component by human bronchial epithelial cells, *in vitro* after a) 1 hour, b) 3 hours, c) 6 hours and d) 24 hours of incubation

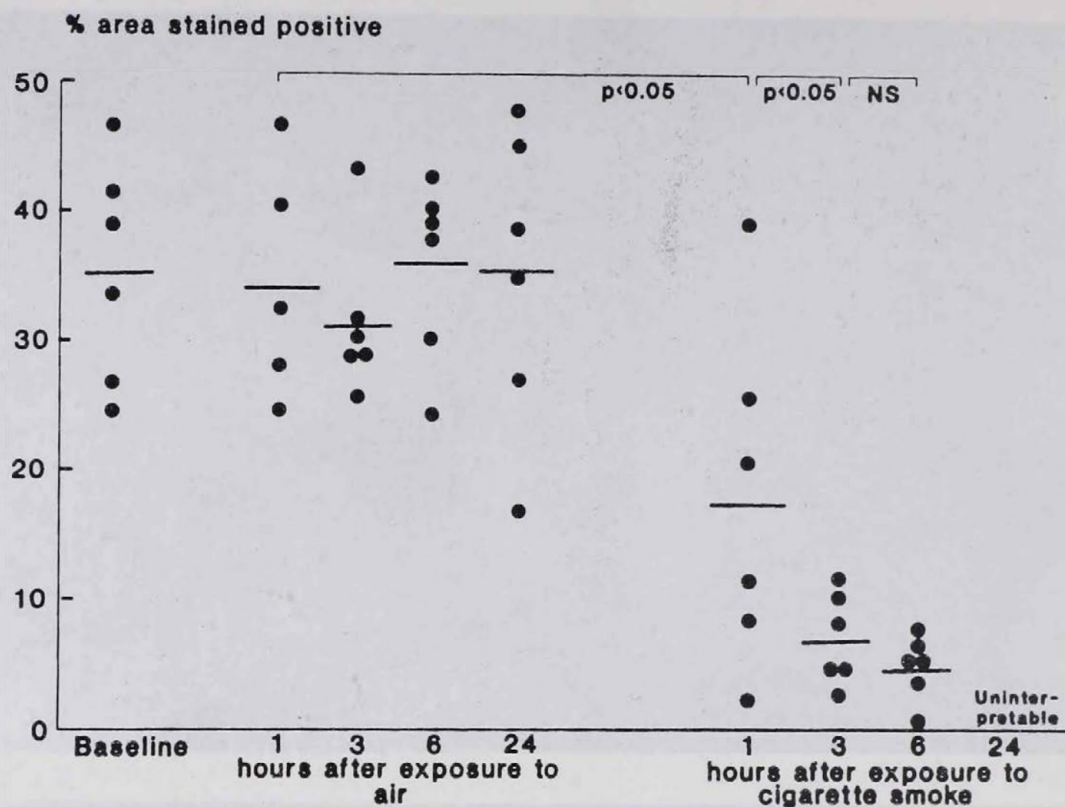


Fig. 4. The effect of 20 minutes exposure to air or cigarette smoke on the expression of secretory component by human bronchial epithelial cells, *in vitro* after a) 1 hour, b) 3 hours, c) 6 hours and d) 24 hours of incubation. In each exposure/incubation group, each data point represents a culture from a separate individual. Bars represent median values. Statistical analysis was performed using Mann Whitney U test.

Taken together, cigarette smoke exposure can lead to alterations in the mechanisms of the host defense system including impaired ciliary function, increased mucous production, increased production of pro-inflammatory cytokines, quantitative and qualitative changes in cellular components and altered humoral response to antigens due to decreased production of SC. These mechanisms are all likely to contribute to the observed increased incidence of respiratory tract infections in individuals exposed to active or passive smoking.

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THE SPREAD AND ANTIBIOTIC RESISTANCE OF *SALMONELLA ENTERICA* SEROTYPE TYPHIMURIUM DT104 IN HUNGARY

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Comparison of phage types (PTs) determined by Felix and Callow's and Anderson's methods was performed testing 99 human strains of *S. enterica* serotype Typhimurium (*S. typhimurium*) isolated in Hungary. PT2 and PT2c – according to Felix–Callow – corresponded with Anderson's DT104 in case of 39 strains out of 40. Among 59 isolates belonging to other Felix–Callow's PTs only one strain was found which was DT 104. Similar unambiguous equalities could not be established between any other PTs comparing the two methods. The PTs of 17877 human strains isolated between 1988 and 1999 were determined using Felix–Callow's method. On the basis of the above equality the emergence of DT104 could be followed retrospectively by means of the rate of PT2 and PT2c. The increase of DT104 began already in 1989, emerging first PT2c then PT2. It predominated since 1991 and it reached its maximum (78.3%) in 1999. The incidence of multiresistance among one of the groups of DT104 strains (Felix–Callow's PT2) was significantly higher in 1998 than the average of non-DT104 strains. The predominant R-type was ACST.

Keywords: phage types, antibiotic resistance *Salmonella enterica* serotype Typhimurium

An increasing incidence of *S. enterica* serotype Typhimurium (*S. typhimurium*) definitive type 104 (DT 104) was observed among the strains isolated from cattle in England, Wales and Scotland since 1991; DT104 became the most frequent PT in 1992; it was first found among the human isolates in 1984 [1]; its spread from cattle to man was proved in 1995 [2]. It has accounted for up to 16% of human isolates in Scotland [3] and it was the most frequent PT originated from farm animals in 1996 [4]. It has spread as a new epidemic strain efficiently through the food chain in the USA

[5], Canada [6] and Europe [7], and it became the second most prevalent salmonella PT in human infection reported worldwide [8]. Interestingly, it was rare in Japan [9].

DT 104 could be characterized with relatively high frequency of multidrug resistance; in 1992 49% of the DT104 isolates from cattle were multiresistant [1]. The original R-type was ACSSuT, which was predominant later on [10]; this was supplemented with trimethoprim [11] or ciprofloxacin [10] resistance. In South-Israel R-type ACT was observed in 1994; two years later 27% of the isolates belonged to R-type ACTN [11].

The spread of DT 104 in Hungary could not be estimated till now because the Felix and Callow's [12] phage typing method was introduced [13] and used since 1960 and not the Anderson's method [14] used in the Western countries. For this reason it was necessary to compare these methods.

In this respect the first publication was the work of Szmollény et al. [15] dealing with 20 strains of animal origin, having R-type ACST. An equivalence was established between the Felix–Callow's PT-s 2, 2c and Anderson's DT 104.

In the present work this comparison was carried out with 99 human *S. typhimurium* strains isolated in 1998–1999. On the basis of the correlation between PTs, the rate of DT104 could be retrospectively followed among the human *S. typhimurium* strains isolated in 1988–1999 with great probability, and its spread in Hungary could be verified.

Methods

Bacterial strains

The salmonella strains of various serotypes isolated between 1978–1999 were got from the County Institutes of Public Health and Medical Officer Service. Out of them, the biotypes and phage types with Felix–Callow's method and the antibiotic resistance pattern of 17877 human strains of *Salmonella typhimurium* isolated in 1988–1999 were continually determined. For comparative phage typing 99 strains isolated in 1998–1999 representing every Felix–Callow's PT in this period were chosen. All of them had biotype 3. Their distribution on the basis of their antibiotic resistance is presented in Table I.

Phage typing

The phage sets of Felix and Callow's [12] and Anderson's [14] were applied. The adaptation of the Felix–Callow's method and the supplementation of the phage set

was performed by Milch et al. [13]. The PTs obtained with the Anderson's method were reproduced in case of 45 strains by K. Speed in the Central Veterinary Laboratory (Addleston, UK).

Biotype determination was carried out according to Milch et al. [16].

Table I

Resistance and Felix-Callow's phage types of human Salmonella typhimurium strains chosen for comparative phage typing

Resistance to drugs	Felix-Callow's phage types											Total
	1	1a var1	1b	2	2a	2b	2c	2d	4	35	Nt	
0	1	2			1	4	5	4	2	4	3	26
1 or 2				3	1		2	4		1		11
3				3		1	2	1				7
4 or more	4		2	14	1	2	11	1	2	18		55
Total	5	2	2	20	3	7	20	10	4	23	3	99

Antibacterial susceptibility testing

Isolates were tested with the disk diffusion method on Mueller-Hinton agar (Oxoid). The antibiotics tested included streptomycin (S) 30 µg, chloramphenicol (C) 30 µg, gentamycin (G) 20 µg, ampicillin (A) 20 µg, kanamycin (K) 30 µg, nalidixic acid (N) 30 µg, sumetrolim (Sxt) 25 µg, cephaperazon (Cfp) 75 µg, cephalixin (Cfl) 30 µg, cefuroxim (Cxm) 30 µg. The disks were from Human (Gödöllő, Hungary) except Cfp, Cfl and Cxm, which were from Oxoid.

Results

The incidence of the most common salmonella serotypes among the Hungarian human isolates in 1978–99 is presented in Fig. 1. In the first period (1978–79) the rate of *S. typhimurium* strains was the highest. After 1984 its frequency significantly lagged

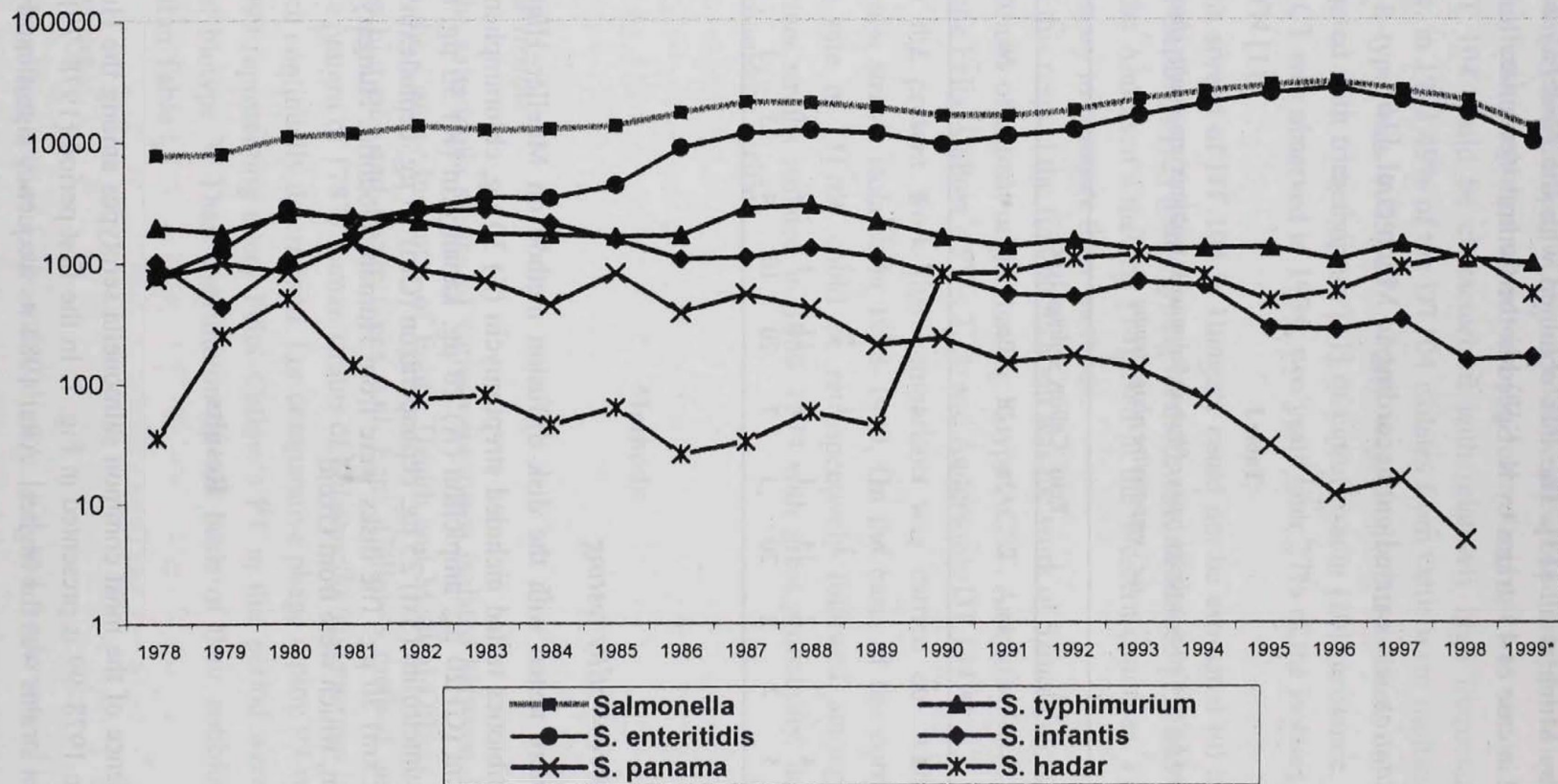


Fig. 1. Frequent human *Salmonella* serotypes in Hungary, 1978–1999
* isolated between 1. January – 30. September

Table II

Phage typing of 99 selected human *S. typhimurium* strains with Felix–Callow's and Anderson's method

Felix–Callow's method	Anderson's method																		
	DT104	Non-DT104																	
		DT 1	DT 8	DT 29	DT 30	DT 73	DT 92	DT 93	DT 99	DT 110	DT 120	DT 126	DT 131	DT 193	DT 203	U 302	NC ¹	UNTY ²	Total
1	1										1						3		5
1a var1													1				1		2
1b																	2		2
2	20																		
2a	1																2		2
2b				1		1	1			1				2				1	7
2c	19					1													1
2d			2				1							1			6		10
4			1		1											1		1	4
35				1				1	1					4	1		2	13	23
Nt				2												1			3
Total	40	1	1	2	5	1	2	1	1	1	1	1	1	7	1	2	16	15	59

¹non characteristic

²untypable

behind *S. enteritidis*, but it was the second one up to the present; it was preceded only in 1998 by *S. hadar*.

The Felix–Callow's and Anderson's PTs of 99 human strains isolated in 1998–99 were compared (Table II). Among 20–20 Felix–Callow's PT2 and PT2c strains, all were proved to be Anderson's DT104 only with one exception; among 59 strains belonging to other Felix–Callow's PTs only one was DT 104. This equivalence was not influenced by the antibiotic resistance. The most frequent DT 104 subtype was DT104 L which was represented by 28 strains among 40 DT104 strains; other subtypes were B, H and I. The other Felix–Callow's PTs distributed among 15 Anderson's PTs without being any exclusive correspondence to any directions. Thirty-one strains were non-characteristic or untypable.

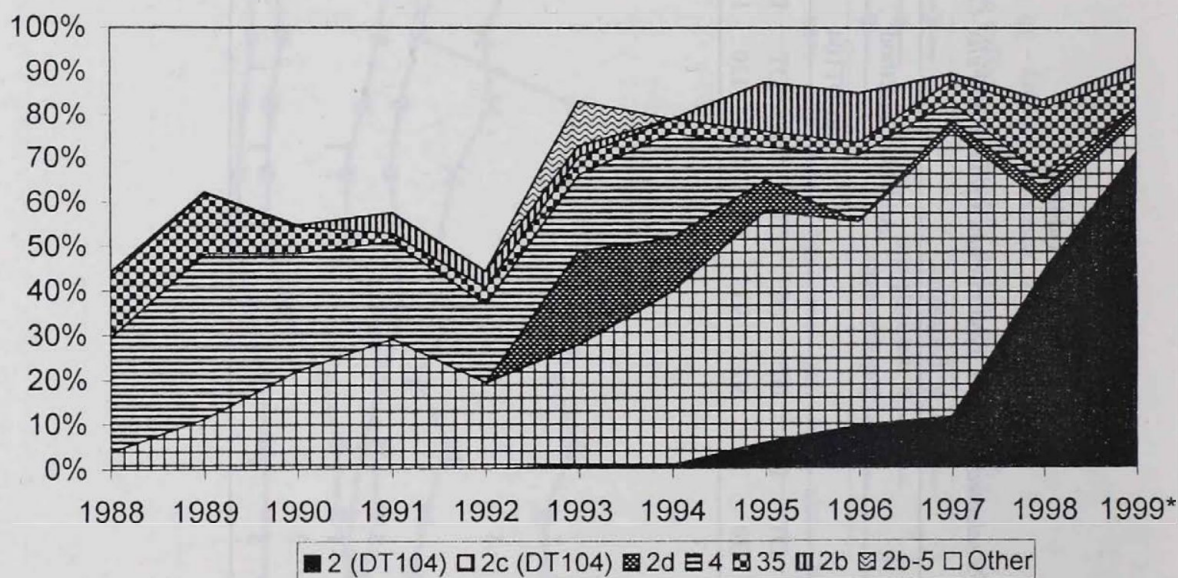


Fig. 2. Phage type distribution of humans *S. typhimurium* strains between 1988–1999

* isolated between 1. January – 30. September

The rates of the Felix–Callow's PT of human isolates in the years 1988–99 are summarized in Fig. 2. Applying the above equivalence it could be supposed with great probability that the strains of Felix–Callow's PTs 2 and 2c which were not phage typed with the Anderson's phage set belonged to DT 104. On the basis of this supposition it was established that the spread of DT104 began already in 1989, it predominated since 1991 and its highest frequency was observed among the strains isolated between

Table III

R-types of human *S. typhimurium* strains isolated between 1. 03 – 31. 12. 1998

S	C	T	A	K	G	N	Sxt	Cfp	Cfl	Cxm	DT104	Non-DT104	Total
											61	104	165
											2		2
											6	1	7
											1		1
											1		1
												3	3
											1		1
											1	1	2
											1	2	3
											2		2
												6	6
												1	1
											26		26
											8		8
											1		1
												1	1
												1	1
											135	4	139
											6	1	7
												1	1
											3		3
											1		1
											25	1	26
											1	1	2
											26		26
												1	1
												1	1
												6	6
												1	1
												1	1
												1	1
											1		1
												1	1
												1	1
											5		5
											1	1	2
												1	1
												9	9
												12	12
												1	1
												33*	33
												1	1
											1	8	9
											316	207	525

Abbreviations: S: streptomycin, C: chloramphenicol, T: tetracycline, A: ampicillin, K: kanamycin, G: gentamycin, N: nalidix acid, Sxt: sumetrolim, Cfp: cephalperazon, Cfl: cephalixin, Cxm: cefuroxim.

*the strains originating from one focus

1. Jan.–30. Sept. 1999 (78.3%). Within the DT104, first the rate of Felix–Callow's PT2c increased while PT2 began to emerge only in 1995, and it became predominant in 1998. The R-types of 523 strains isolated between 1.03–31.12 1998 are shown in Table III. Among 22 R-types exhibited by DT104 strains ACST was the predominant preceding R-types ACSTN and ACSTCfp. The non-DT104 strains are distributed among 29 R-types, but there were no predominant one (not regarding the strains originated from one focus).

The rate of multiresistant strains was much higher and the number of sensitive isolates was lower among DT 104 than the average of other Anderson's phage types (Table IV). This tendency continued also in 1999 (data not shown).

Out of the two groups of DT 104, only the Felix–Callow's PT2 exhibited high rate of multiresistance, this was not observable in case of PT2c.

Table IV

The frequency of multiresistant human S. typhimurium strains depending on the phage type

Resistance	Anderson's DT104			Anderson's non-DT104	Total
	Felix-Callow's		Total		
	PT 2	PT 2c			
Sensitive	2 (0.8)	59 (67.0)	61 (19.3)	104 (50.2)	165 (31.5)
Resistant to 1 or 2 drugs	12 (5.2)	3 (3.4)	15 (4.7)	14 (6.7)	29 (5.5)
Multiresistant	214 (93.8)	26 (29.5)	240 (75.9)	89 (42.9)	329 (62.9)
Total	228	88	316	207	523

Count (%) of human *S. typhimurium* strains isolated between 1.03.–31.12. 1998

Discussion

The importance of *S. typhimurium* in the human salmonellosis in Hungary is clear from Fig. 1, similarly to the worldwide situation. DT 104 became its predominant phage type in the nineties. Felix–Callow's PT2c played an analogous role in Hungary,

but their identity was questionable; this question became more complicated by the increase of PT2 in the last years.

The phage typing method of Felix and Callow [12] was introduced in Hungary in 1960 [13], much earlier than the Anderson's method [14] was published. It was proved to be very suitable for the epidemiological practice owing to its simplicity and unambiguity. On the other hand, the introduction of Anderson's method was opposed by its great work demand, the uncertainties of the evaluation and above all that the phage set could be propagated only in Colindale.

The comparative phage typing which was necessary to solve the above question was carried out with strains represented by 11 Felix-Callow's PT-s occurring in that period and the number of PT2 and 2c was high enough to prove their equality with DT 104. The sufficiently strict correspondence made it possible to follow retrospectively the increase of DT104 in Hungary which took place roughly parallel with its West-European spreading. Interestingly, the two groups of DT104 (Felix-Callow's PT2c and 2) spread in two phases.

The subtyping of the predominant DT 104 by means of simultaneous application of both methods may be useful in the epidemiological practice.

The unusual high rate of multiresistance of DT 104 was reported by the earliest publications already with a characteristic R-type ACS SuT [1, 10, 17–19].

Among 302 strains of mainly animal origin there were no sensitive ones and they were resistant to 4 or more antibiotics in 98% (Low et al. (19)). On the other hand, a striking high proportion of multiresistant salmonellas was DT 104 in the USA (Glynn et al.) [20].

In the present work similar results were obtained only with one group (Felix-Callow's PT2) of our DT104 strains which exhibited high rate of multiresistance; PT2c predominated till 1997 without exceeding the multiresistance rate of non-DT104 isolates. These results indicate that the multiresistance was not an essential factor of the spread of DT 104.

According to our data the predominant R-type was ACST instead of ACSSuT since sumetrolim was applied instead of sulphonamid in the disc test.

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INTERACTION OF IMMUNOSUPPRESSIVE DRUGS IN MOUSE EXPERIMENTS WITH SPECIAL REGARDS TO BACTERIAL TRANSLOCATION

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Following intraperitoneally (i.p.) applied treatment with 12.5 mg/mouse prednisolone (PRD) no bacterial translocation (BT) was observed in mice. The PRD treatment applied in combination with lymphotropic cytostatics as dianhydrogalactitol (30 mg/kg i.p.) or chlorpromazine (75 mg/kg i.p.) both causing BT, did not increase the mice's drug sensitivity to the used agents. According to our results, PRD can be suitable for combined application with other immunosuppressive agents as it can increase immunosuppression without increase of side-effects such as those induced by bacterial translocation.

Keywords: immunosuppressive drugs, bacterial translocation, cellular immune response

It is known that in certain immunosuppressive conditions bacteria of the normal intestinal flora may permeate the intestinal wall and can be translocated into the otherwise sterile inner organs [1]. In our earlier experiments, following the cytostatic dianhydrodulcitol (DAD) or chlorpromazine (CPZ) treatments bacterial translocations (BT) and simultaneously enhanced sensitivity to the applied agents as compared to germfree mice were observed in conventional mice [2, 3]. The drug sensitivity increased also after combined use of the two agents [4]. It is also known from published data and learned from our experiments, too that both agents have definite adverse effects on the intestinal wall, and presumably the BT appearance and the increased drug sensitivity are consequence of the intestinal wall injuries [4–6].

In the present study we looked for immunosuppressive agents without any effect on the intestinal mucosa. First, antilymphocyte serum (ALS) was examined. According

to our results, following the ALS treatment no BT and consequent endotoxin effect developed. Applied in combination with cytostatic dianhydrodulcitol or chlorpromazine, the ALS did not aggravate their adverse effects on the intestinal wall and thus did not cause enhanced drug sensitivity [7].

In the experiments we are going to present the effects of prednisolone (PRD) were examined. From human therapy the PRD is a well-known immunosuppressive agent, and its adverse effect on the immune system in animal experiments is known [8]. We tried to find an answer as to whether

- BT can be identified in immunosuppressive state following PRD treatment,
- PRD treatment can induce changes in drug sensitivity when used in combination with other immunosuppressive agents.

Materials and methods

Experimental animals

Six to eight-week-old young adult conventional CD-1 mice of 23–25 g weight (Charles River Hungary Kft) of both sexes were used.

Prednisolone treatment

Di-adreson F aqueous injection (Organon) containing 25 mg prednisolone nitrate succinate per ampoule was used. Mice were inoculated intraperitoneally (i.p.) with 12.5 mg PRD in 0.5 ml on one occasion.

Dianhydrogalactitol treatment

Dianhydrogalactitol (DAG) which is epoxide of dibromdulcitol (DBD) and stereoisomer of dianhydrodulcitol (DAD) is a lymphotropic cytostatic agent in the alkylating group (Chinoin Pharmaceutical Works Ltd., Budapest). The substances were dissolved in sterile distilled water and used within half an hour. Dilution was made and 0.01 ml/g body weight was inoculated i.p. on one occasion.

Chlorpromazine treatment

A 2.5% aqueous solution of chlorpromazine hydrochloride (EGIS, Budapest) was used. The proper dilution was prepared with PBS, immediately before use and 0.01 ml/g body weight was inoculated i.p. once.

Control groups' treatment

Control mice (Group C) were inoculated i.p. by sterile PBS of 0.5 ml/mouse.

Examination of bacterial translocation

Preparation of cell suspension: After the mice were killed, two mesenteric lymph nodes, the spleen and the liver were removed with sterile instruments. Cell suspensions were prepared in 0.5 ml PBS individually from the removed lymph nodes as well as from the fragments of 100 mg of spleen and liver, respectively.

Isolation of bacteria

The whole quantity of each of the cell suspensions was inoculated into serum bouillon and incubated at 37 °C. Subcultures were made of the serum bouillon on blood agar medium at 24 h intervals and they were aerobically incubated. Isolations were considered negative when no bacterial growth was observed on the blood agar media after 48 h incubation. Inoculations were repeated daily on five successive days.

Lymphocytic choriomeningitis (LCM) virus infection

The applied W.E. strain is maintained in serial mouse brain passages. In every case sterile PBS solution was used to prepare brain-suspension and virus dilution. Experimental animals were inoculated intracerebrally (i.cer.) with 0.03 ml of pretitrated 100 LD₅₀ of LCM virus. Control animals received non-infected brain-suspension in the same way. During the experiments, the development of neurological symptoms characteristic of LCM (tremor, convulsions) was controlled twice daily, and mortality was registered.

Lymphocyte count

It was determined in caudal vein samples under standardised conditions.

Examination of the lymphoid system

The relative spleen and thymus weights were determined as follows:

$$\text{Relative lymphoid organ weight} = \frac{\text{lymphoid organ weight (mg)}}{\text{body weight (g)}}$$

Statistical evaluation

It was carried out by the use of T-probe through Microsoft Excel, and a significance level of $p=0.05$ was accepted.

Experiments and results

From examination of the peripheral lymphocyte counts (a) and lymphoid organ weights (b) as well as the cellular immune response to the i.cer. LCM virus infection (c), we concluded to their immunosuppressive effects on mice.

(a) Lymphocyte counts were determined on the 3rd and 7th day after PRD treatment of the treated and control groups of 6–6 mice. Table I shows that significant lymphopenia is caused by the applied PRD treatment, that can be observed more definitely on the 3rd day after treatment and still observable on the 7th day.

(b) To examine the lymphoid organs, 12–12 mice were inoculated with PRD or phosphate buffered saline (PBS) and then following the treatment 6–6 treated and control mice were killed on the 3rd and 7th day and relative lymphoid organ weights were calculated. As it turns out from the results, no changes are observed in the relative spleen and thymus weights either on the 3rd or the 7th day.

(c) To determine the PRD effect on the cellular immune response decrease it was examined how PRD affected LCM development after i.cer. LCM virus infection [9, 10]. Ten-ten PRD treated and non-treated mice were inoculated i.cer. with pretitrated 100 LD₅₀ of LCM virus.

Hundred per cent of mice infected with 100 LD₅₀ of LCM virus died on the 7–9th day following the i.cer. infection amidst neurological symptoms characteristic of LCM (tremor, convulsions), while 50% of PRD treated animals survived and no LCM developed. This clearly shows the cellular immune response decreasing effect of the PRD treatment applied.

Two experiments were conducted to answer the questions raised in the introduction.

Experiment 1. Groups of 18–18 mice were treated with PRD or PBS as a control. We carried out daily isolations of bacteria from the mice organs on the first, the second and the third day after the treatment, killing 6 mice both from the PRD treated (PRD) and control (C) groups, to study BT (Table II).

In this experiment of three successive days we could not observe BT, either in the PRD or C groups (Table II).

Table I*Lymphocyte count (ly) averages on the 3rd and 7th day after PR treatment*

Groups	Ly averages	Standard deviation	p value
PRD/3	2000	± 201	< 0.001
C/3	5900	± 760	
PRD/7	3200	± 668	< 0.001
C/7	5900	± 383	

Table II*Frequency of bacterial translocation in Experiment I*

Groups of mice	Treatments i.p.	Examination after treatment on	Translocation frequency*	
			in mice	in organs
PRD	12.5 mg	1st day	0/6	0/18
		2nd day	0/6	0/18
		3rd day	0/6	0/18
C	0.5 ml PBS	1st day	0/6	0/18
		2nd day	0/6	0/18
		3rd day	0/6	0/18

*Number of positive mice or organs / Number of examined mice or organs

Experiment 2. Further, we applied PRD pretreatment in combination with immuno-suppressive agents and examined whether PRD treatment could change drug sensitivity. On the day after PRD treatment we inoculated mice's groups with subtoxic doses of DAG or CPZ, namely with 30 mg/kg DAG or 75 mg/kg CPZ (CPZ LD50: 100 mg/kg, DAG LD50: 35 mg/kg). The observation period lasted 21 days.

Table III shows the groups established and the number of mice in each group as well as the treatments applied and mortality.

From this table it can be seen that PRD pretreatment that caused no mortality by itself (PRD group), did not affect the mortality rates caused by DAG or CPZ (PRD-DAG, or PRD-CPZ groups) and consequently did not lead to enhanced drug sensitivity to immunosuppressive agents.

Table III

Groups of mice, number of mice as well as the applied treatments and mortality rate in Experiment 2

Groups	Number of mice	I.p. treatments				Mortality rate
		PRD (mg/mouse)	DAG (mg/kg)	CPZ (mg/kg)	PBS (ml)	
PRD	20	0.5	–	–	–	0/20
DAG	20	–	30	–	–	2/20
PRD-DAG	20	12.5	30	–	–	2/20
CPZ	20	–	–	75	–	7/20
PRD-CPZ	20	12.5	–	75	–	7/20
C	20	–	–	–	0.5	0/20

Discussion

The immunosuppressive effect of DAD has been proved in our previous investigations [10]. BT caused by DAD and DAG, its stereoisomer, has been demonstrated as well after a single large dose or chronic treatment [5]. The BT occurrence is due to the cytostatic effect of the agent and connected at the same time with its adverse effect on the intestinal mucosa [2]. It is known from literature and our results, too that CPZ has an immunosuppressive effect and further induces intestinal atony as well as consecutive increased permeability [3, 6]. Similar drug sensitivity developed when we used the two agents (DAG, CPZ) in combination [4].

In our present experiments we investigated the effect of PRD on BT. PR has no adverse effect on the intestinal mucosa. This may explain our results that no BT and consecutive endotoxic effect were experienced as well as PR did not increase the adverse effect on the intestinal wall of the agents – DAG or CPZ in our case – used in combination, i.e. no increased drug sensitivity developed [5]. The experimental results correspond to those received in connection with ALS treatment.

In previous experiments we have demonstrated that mice with impaired lymphoid system show an increased sensibility to further lymphoid system damaging interventions. For example in case of combined use of cytostatics the lymphoid system and/or the intestinal mucosa damaging effects are summed up [11].

From the present results one can conclude that PRD – similarly to ALS – seems suitable for combined use with immunosuppressive agents because immunosuppression can be increased without increasing adverse side-effect induced by BT.

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THE PLACE OF VIRUSES IN THE “TREE OF LIFE”*

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Ribozymal entry into vesicle containing autocatalytically replicating oligopeptides engendered RNA proliferation and enzyme synthesis within units whose RNA genomes derived from ancestors of viroids.

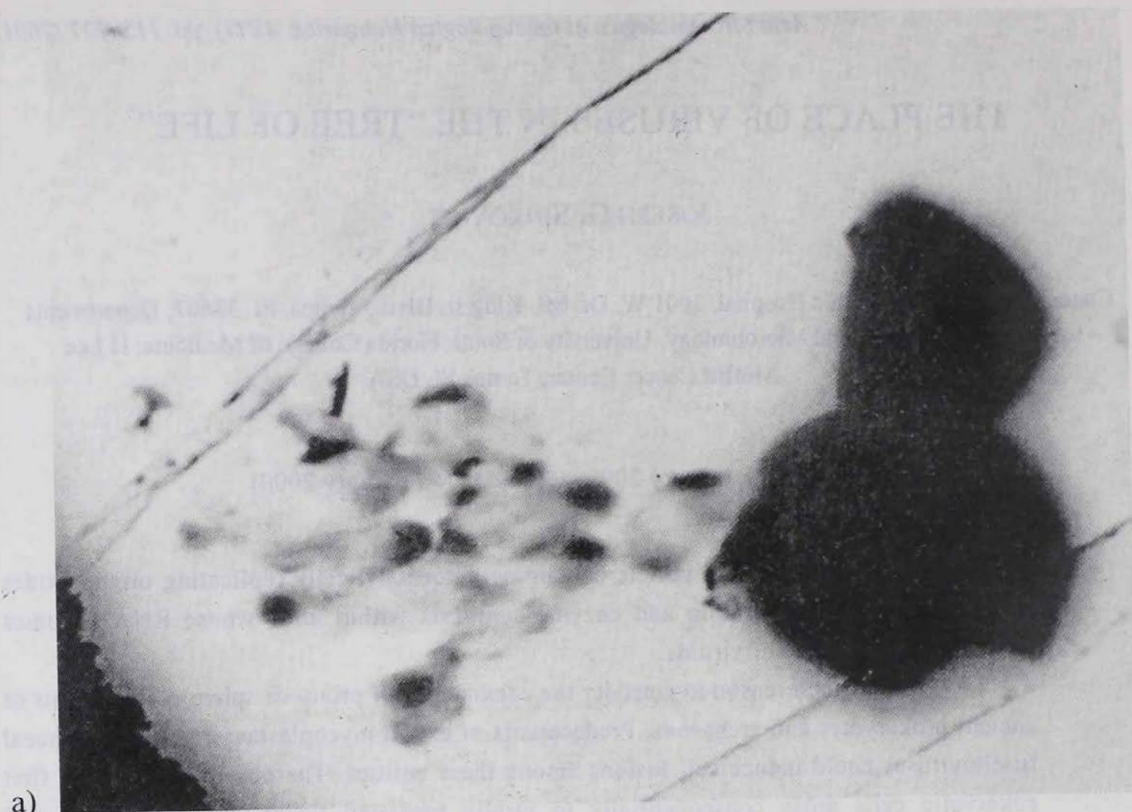
There is good reason to consider the coexistence of proto- or spheroplastic forms of ancient prokaryotes and archaeons. Predecessors of extant mycoplasma virus L3 or archaeal fuselloviruses could induce cell fusions among these entities. The possibility that the first eukaryotic cells arose consequentially to virally mediated fusions of prokaryotic and archaeal proto- or spheroplasts is presented. Retrotransposons and endogenous retroviruses might have emerged in theropod dinosaurs when *Aves* evolved; and directed the development of syncytiotrophoblasts in the placenta of the first mammals. As viruses co-evolved with their hosts descendants of ancient viruses diverged from one another. Certain phenotypical features could connect extant phages and eukaryotic viruses to common ancestors.

Keywords: ribozyme→viroids, retrotransposons→retrotransposons, protocell fusions virally mediated

Virally mediated fusion of ancient proto-spheroplasts

At the Microbial Genome III meeting in Chantilly, Virginia, Dr. R. Gupta's presentation on the chimeric fusion between an ancient prokaryote and archaeon to produce the first eukaryote [1] received “lukewarm reception” [2]. However, envisioning such a spectacular scenario from the point of view of a seasoned virologist

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is not difficult at all. Long ago we observed that rigid cell wall-free proto- or spheroplastic units of an *E. coli* strain (Figure 1a, b) passed collodion membrane filters and regenerated into vegetative bacterial cells after 350 m μ filterable units fused with one another [3, 4]. While this observation remained incomplete, it received immediate favorable attention [5, 6]. It established that cell wall-free subunits of the same or related bacterial strains or species readily recombine by fusion in their proto- or spheroplastic stage of existence. Cells of *E. coli* operate with 3000 genes within a 4.7 Mbp genome but elementary bodies of *Mycoplasma genitalium* live and replicate themselves with a minimum of 265 to 480 genes [7].

Spontaneous proto- or spheroplast formation is not confined to prokaryotes. Spontaneous or induced proto- or spheroplasts occur among archaeons; for example in *Methanobacterium bryantii* or *Methanospirillum hungatii* [8, 9]. Fusion between cell wall-free proto- or spheroplasts of different orders, families or species was probably uncommon even among archaic life forms (while bacterial geneticists allow so liberally lateral transfers and exchanges of the ancient gene pool among these entities) [10, 11]. However, such fusions could be facilitated by viral agents. Rigid cell wall-free eukaryotic cells readily fuse under the effect of numerous viruses: Sendai, Newcastle disease and human immunodeficiency viruses, etc. that possess fusion proteins and induce the formation of polykaryotes and large cytoplasmic masses [12, 13]. Indeed viral agents fusing rigid cell wall-free bacterial subunits also exist. For example the L3 virus fuses mycoplasma cells thus generating giant cells of this filterable microorganism [14]. Mycoplasma virus MV-L3 (from *Acheloplasma laidlawii*) is a tailed bacteriophage possessing linear dsDNA slightly less than 40 kb each and forming temperature-sensitive mutants that readily recombine with each other [15]. As to rigid cell walls, this presentation is based on the concept that the universal ancestor(s) [16] spent considerable time in a protoplastic-spheroplastic form of existence before they evolved genes for cell wall synthesis as opposed to another concept [17, 18] according to which rigid cell walls existed early in prokaryotes until after one prokaryote produced the first antibiotic (or bacteriocin) that inhibited cell wall synthesis in other prokaryotes possibly for some selection advantage; archaeons do not possess rigid cell walls only rigid cell membranes.

Fig. 1a, b. Malleable bacterial cytoplasm containing electron dense elements protruding from vegetative *E. coli* cells after rupture of the rigid cell wall; 350 m μ units regenerated into vegetative cells after fusion with each other [3, 4]. EM3-1951 Russian electron microscope, transmission photograph, 1:8000–1:10,000 original magnification. (Overdue but not forgotten credit goes to Dr. Ferenc Fornosi for the preparation of filter membranes; to Dr. Iván Hollós for the preparation of the electron microscopic pictures). Reproduced with permission from *Acta Microbiologica Hungarica* (1957).

Viruses as ancient biomolecules

Presumably viral evolution occurred gradually extant viroids being the descendants of the earliest self-replicating RNA molecules that failed to encode peptide chains but already functioned as ribozymes [19, 20]; and RNA viruses derived from the first protein-encoding mRNA molecules [21, 22]. The evolution of enzyme machinery [23, 24] and that of the genetic code [25] was necessary for the transcription of RNA into DNA. While RNA molecules evolve and mutate rapidly (including RNA viruses) [26, 27], DNA is a much more stable depository of genetic information. At this point of evolution compartmentalized primitive cells must have already existed and DNA viruses to begin their slow evolution took their origin from them [28]. In the subsequent DNA world second generation of RNA viruses utilizing reverse transcriptase must have derived [29, 30]. If accepted that mycoplasmas are the result of a retrograde evolutionary process their ancestors being clostridia undergoing genomic attrition [14], then the MV-L3 virus of extant *Acheloplasma* may not be an ancient virus mediating cell fusions early at the root of the tree of life. It only serves as an example of a virus that induces fusion of primitive cells of lipoglycan membranes [31].

May now the presumption be allowed that cell fusion-inducing viruses might have existed among ancient protoplast-like archaeons and prokaryotes? Extant *Fuselloviridae* of Crenarchaeota also possess 16 kb dsDNA (but circular not linear) and highly hydrophobic envelopes [28, 32]. Ancestral *Fuselloviridae* attaching to cell membranes of two or more proto- or spheroplasmic cells may unite these cells into a large conglomerate.

If we consider that the rigid cell wall-free malleable protoplasmic substance exuding from an extant Gram-negative bacterium during its proto- or spheroplasmic life cycle (Figure 1a, b) is similar to the most ancient living matter as it existed at the root of the tree of life, and that the ancestors of prokaryotes and archaeons coexisted once in this life form, an emerging viral agent like that of the extant MV-L3 or archaeal fusellovirus could promote the fusion of these subunits (Figure 2).

Symbiotic-metabolic coexistence and/or mergers of prokaryotic and archaic ancestors to form the first eukaryote have been proposed repeatedly and in many forms [1, 33–35]. The sequence of events concerning the acquisition and loss of rigid cell walls of the prokaryotic partner; or the engulfment of ancient proteobacteria, donors of aerobic mitochondria, in relation to oxygen-production by co-existing chloroplasted eukaryotic plant cells (microalgae); or the time when polyphyletic hydrogenosomes [36] were acquired by ancient protists all remain unsettled and heatedly debated.

The phylogeny of membranes according to Blobel [37] began with the double membrane of fused protocells and with the formation of intracellular compartments

(nuclear membrane, endoplasmic reticulum, ribosomes, lysosomes, Golgi complex, peroxysomes) through invaginations of the internal membrane. Rigid cell walls of prokaryotes must have evolved after long coexistence of the universal ancestors without it.

Coevolution of viruses with their hosts

Viral agents harbored by the protocellular predecessors became parasites of the newly formed eukaryotes. Extant eukaryotic viruses diverged from their ancestors way past recognition of their relatedness as based on their genomic or protein sequences. However, some extant phages and animal viruses display phenotypical features that connect them to a common ancestor [38]. Examples are the dsDNA *E. coli* phage PRD1 and adenoviruses: the structure of their P3 and hexon capsid subunits, respectively; and their β -barrel protein folds remain very similar. Other viruses still sharing the interconnected 8-stranded antiparallel β -barrel are phage Φ X174, TBSV plant virus, SV40, rhino- and polioviruses. Adenoviruses evolved the substrain-variable "tower loops" over their β -barrels that are absent in the β -barreled phages. Host immune reactions that recognize these adenoviral tower loops are nonexistent in the host of these phages. Further structural (nonantigenic) similarities (or relatednesses) between PRD1 phage and adenoviruses concern their capsid pentamer geometry and the inverted terminal repeats of their DNA genomes. Further there are similarities between the mode of assembly of the icosahedral procapsids of coliphage T4 and herpesviruses as these viruses cut their α -helical scaffolding by virally encoded proteases, discard the fragments and package their DNA genomes in the capsid at about the same density: 40 bp/10⁵ cubic Å. Of dsRNA viruses, *Pseudomonas* phage Φ 6 and plant- or animal-infecting reoviruses package their genomes into very similar double shelled capsids. Finally [38], tailed phages that infect both extant prokaryotes (*Bacillus* sp.) and archaea exist. Did the Ψ M2 phage evolve from an ancient virus that was active in the "universal ancestor" before the separation of prokaryotic and archaeal lineages?

Cech's ribozymes are ancestors of extant viroids [19] and indeed must have interacted with autocatalytically replicating polypeptides probably within vesicles of bilayered lipid membranes. Ancient enzymes were formed from oligopeptides deposited on the framework of nucleotides (for example aminoacyl-tRNA synthetase) [23]. Reactions generated in the laboratories of Ghadiri [39] and Eigen [40], respectively approximate most the events occurring at the dividing line between inanimate and living matters.

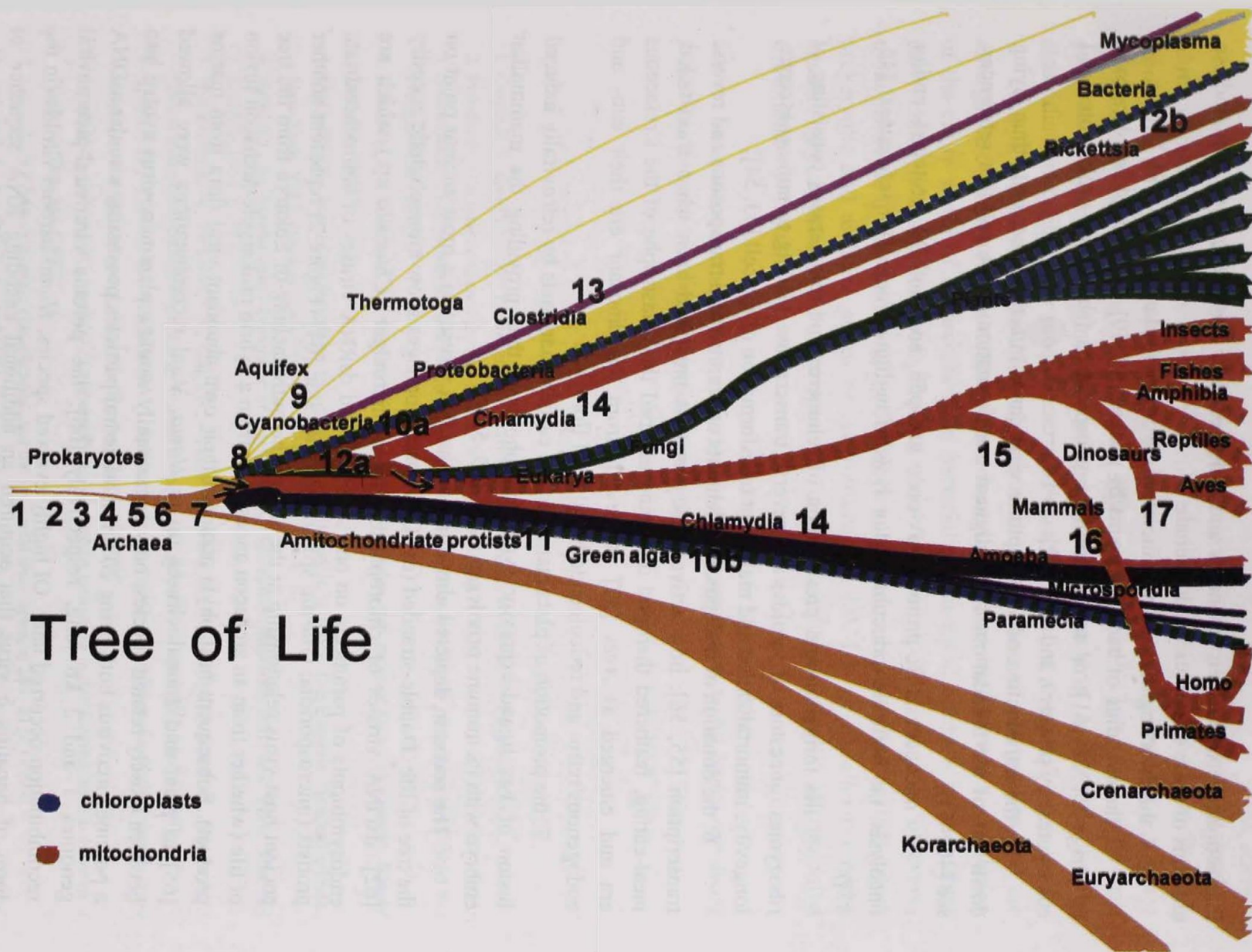
Genomic sequences exchanged between vesicles of bilayered lipid membranes occurred gradually: lateral gene transfers in the form of 1. naked RNA and later DNA; 2. plasmids; and 3. packaged into capsids. Acquired alien genomic sequences either replicated themselves in their new hosts or were incorporated in the new host's genome. In the latter case, Lwoff's lysogeny could be activated by UV light exposure; or retrotransposons were formed eventually giving rise to retrolentiviruses: from plant pararetroviruses and animal hepadnaviruses up to ancient human endogenous retrolentiviruses encoding a functional homolog of the HIV-1 REV protein [41–43]. The endogenous human HERV-K retroviral sequences were present in the simian genome predating the divergence of early hominoids from Old World monkeys [44]. The HERV-K-T47D sequences are functional in the human placenta and in certain breast cancers [45].

Viruses not only destroy their host cells as they reassemble their new progenies; viruses have powerfully influenced the evolution of their hosts in a constant flux and

→

Fig. 2. Theoretical reconstruction of the "tree of life": proto- or spheroplastic cells of prokaryotes and archaeons coexist (1st billion years) and first viruses are formed. Chimeric fusion products of prokaryotes and archaeons engender first eukaryotes; this fusion was mediated by an ancient virus (2nd billion years begin)

1. Universal ancestor [16]; 2. Bacterial-archaeal biochemistry [28, 69–71]; 3. Origin of RNA replicons, viroids and their satellites [19, 72]; 4. 1st generation RNA viruses (additional RNA sequences translated into polypeptides) [72]; tRNA [23, 73, 74]; 5. DNA and its enzymes [75]; genetic code [25, 76]. First DNA viruses [30]. Telomeres and telomerases (ribozymes and reverse transcriptase) [77, 78]; 6. 2nd generation RNA viruses (retrotransposons) [72]; recombinant RNA viruses [79]; 7. Lateral gene flow and exchange of genomes [10, 11] by naked DNA or by virus- (phage-) mediated transduction-transfection [80]; 8. Virally mediated fusion of prokaryotic and archaeal proto- or spheroplasts: the origin of eukaryotes (without rigid cell walls) (for references [46, 47]); 9. Rigid cell wall synthesis (and sporulation?) among Bacteria; 10. Cyanobacteria (a) donate chloroplasts [81]: evolution of chloroplasted green microalgae (b) and their dsDNA viruses as they become endosymbionts of eukaryotic paramecia. Evolution of marine and terrestrial plants and plant viruses [82]; 11. Amitochondriate early eukaryotes: microsporidia (*Giardia* sp.); protists; ciliates; paramecia and their dsRNA viruses (for references [18]); 12. Proteobacteria (a) donate mitochondria: eukaryotes acquire mitochondrial genes→"Cambrian explosion." Proteobacteria evolve into rickettsiales (b) (for references [18, 83]); 13. Clostridia (a): one branch through genomic attrition becomes *Acheloplasma* and *Mycoplasma* [14]; 14. Prochlamydiae become endosymbionts of *Acanthamoebae* (for references [46, 47]); 15. Endo-exogenous retroviruses mediate fusion of syncytiotrophoblasts in early placentation of emerging mammals [58–60]; Mammalian host cell mRNA contributes to the formation of enterovirus (poliovirus) [21]; 17. Transposable elements (retrotransposons) activated as birds evolve from theropod dinosaurs; 18. Emergence of lymphocytotropic herpes, endo- and exogenous retrolentiviruses in the simian-hominoid-*Homo* lineages that coevolved in Africa replacing interference with mutual transactivation for coexistence (for references [46, 47, 84–86]) (not shown).



interactions between their genomes and those of their host cells [46, 47]. Endless citations of these examples would include

1. the dwarfing of hosts with increasing fruition by plant viroids [48];
2. the encoding of bacterial toxins by phages [49, 50], for example cholera toxin by phage CTX Φ [51]; or serine-threonine phosphatases (shared by prokaryotes and eukaryotes) by phages λ and Φ 80 [52];
3. the introduction of antiapoptotic viral genes into their host cells thus laying down one of the foundations for malignant transformation of the cell (for references: see [46, 47]);
4. oncogenesis (c-protoonc $\rightarrow$ v-onc; anti-p53 and anti-Rb proteins) and/or oncolysis, i.e. both the induction and/or lysis of malignant cells (for references: [46, 47]);
5. the formation and preservation of telomeres and telomerases consisting of ribozymes (ancestors of viroids) and reverse transcriptase leading to anti-senescence, longevity, immortalization and malignant transformation of the cell [53, 54];
6. the donation of transposable elements or prero-viral transposons and reverse transcription [55, 56]: how active these elements must have been when short-tailed, meat-eating, feathered theropod dinosaurs escaped the catastrophe of the Cretaceous era and emerged as *Aves* [57] preserving from the dinosaur era their exo- and endogenous retro- and reticuloendothelial viral flora; and
7. the promotion of placentation in the earliest mammals by retrovirally induced fusion of Fas ligand-expressor syncytiotrophoblasts thus providing the mammalian embryo with its immune privileged status [55, 58–61].

The scenario depicted above places the first viruses to a most ancient point on the tree of life. Double-stranded (ds) DNA bacteriophages show monophyletic ancestry [62]. dsDNA viruses of chloroplasted green microalgae (*Chlorella* sp.) which are endosymbionts of paramecia on one side [63] and dsRNA viruses of amitochondriate protists (microsporidia; *Giardia* sp) on the other side [64] (Figure 2) represent another ancient host-virus relationship at the site of the outbranching of Eukarya from the tree of life (whether from an archaeon ancestor or from a hybrid prokaryote-archaeon fusion product). Subsequent host phyla acquired their own abundant viral flora from marine [65] to plant and animal viruses up to *Homo*. Viral recombinations were allowed between closely related species; only occasionally would a plant nanovirus evolve into a porcine circovirus both being 20 nm icosahedral particles possessing circular ssDNA genomes 1 and 2 kb long, respectively [66]; the porcine circoviral-picornaviral recombination occurred later. Of highly evolved species, *Homo* harbors viroids (in the form of hepatitis δ virus that acquired an additional encoding RNA sequence of probable host derivation) [67]; simple enteroviruses (poliovirus) probably deriving

from host cell mRNA [21]; and complex retrolentiviruses deriving from ancient retrotransposons [68]. The complex DNA viruses (variola; herpesviridae) through "molecular piracy" enriched their genomes with acquisitioned ancient host cell genes of basic biological functions. Could some of these latter viruses have derived through genomic attrition from some ancient endosymbiotic bacteria analogously to the clostridia→mycoplasma or proteobacteria→rickettsia sequence of events?

It is not only the evolution of *Aves* or the acquisition of placentation and the emergence of mammals that depended on retroviruses; it may well be that the evolution of the entire world of eukaryotes from bacterial and archaeal ancestry through their early chimeric fusion had been brought about by a virus. This sequence of events monumental as it may seem could probably be subjected to experimental testing: extant prokaryotic and archaeal proto- or spheroplasts and MV-L3- or *Fuselloviridae*-like agents may engender the formation of chimeric eukaryotic cells of unlimited evolutionary potential.

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DETECTION AND TOXIN PRODUCTION OF *STAPHYLOCOCCUS AUREUS* IN SUDDEN INFANT DEATH CASES IN HUNGARY*

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The potential role of microbial agents was investigated in 13 cases of Sudden Infant Death Syndrome and in 9 non-SIDS cases in Budapest between September 1996 and May 1998. Autopsy, histological examination and microbiological tests were performed on samples of blood, cerebrospinal fluid, pharyngeal samples and lung tissue from infants under one year died suddenly, without previous diseases. The multifactorial pathomechanism of SIDS was suggested by the isolation of toxin producing *Staphylococcus aureus*-, *Enterobacteriaceae* and *Candida albicans* strains in large number and by the detection of Parainfluenza Type 2 virus antigen. *S. aureus* proved the predominant bacteria in the SIDS cases. Nasopharyngeal microbial flora and *S. aureus* carrier of 100 age matched healthy infants were tested during the same period. *S. aureus* was isolated from 54% of SIDS cases and 37% from healthy infants /OR=1.986 (95% Confidence interval=0.55–7.33), p=0.243/. The enterotoxin and TSST-1 toxin producing activity of *S. aureus* showed the characteristic difference. The toxigenic *S. aureus* was detected in 46% of SIDS cases and 16% of healthy infants /OR=4.5 (95% CI=1.15–17.72), p=0.010/. The distribution of toxigenic and nontoxigenic isolates was 86% in SIDS cases and 43% in healthy infants /OR=7.875 (CI=0.78–191.89), p=0.041/.

Keywords: sudden infant death syndrome, bacterial toxin production, *Staphylococcus aureus*

Sudden Infant Death Syndrome (SIDS) is an outstanding problem in Hungary. There has been a significant decrease in infant mortality rate in the recent decades; however, the incidence of SIDS did not decrease. Between 1980–1996, the SIDS

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incidence was 0.12–0.36 per 1,000 live births [1]. Infectious agents, bacterial exo- and endotoxins can be triggers for sudden infant death. Bacterial toxins exert a powerful effect on the immune system, stimulating T cells, and subsequently induce the formation of large amounts of cytokines. Generation of overwhelming inflammatory response may lead to death by the shock of the cardiac or respiratory system [2].

The purpose of our study was to investigate whether there are correlations between the pathological changes of microbial flora and SIDS.

Materials and methods

Thirteen infants who died of SIDS between September of 1996 and May of 1998 in Budapest and 9 age matched infants, under one year who died unexpectedly or in accidents in Budapest were examined by detailed autopsy, histological and microbiological tests. Nasopharyngeal microbial flora and isolation of *S. aureus* from 100 under one year old infants were tested during the year 1997 (January–March 24 infants, April–June 36 infants, July–September 20 infants, October–December 20 infants). The healthy infants under one year of age were randomly selected from infants considered healthy after a regular control from 3 different primary health care practices, Budapest.

Methods

Medical data of SIDS and non-SIDS infants were collected through the collaboration of parents and pediatricians. (Permission of the Ethical Committee: No: 32/1998.)

Autopsy and histological tests

Samples from various organs (heart-blood, lung, liver, spleen, kidney and segments of small and large intestine) were obtained during the autopsies. The autopsy was done within two days of sudden death. The bodies were stored at 4 °C before the autopsy. Mild infections of the upper respiratory tract or middle ear, or positive bacteriological or virological findings unaccompanied by morphological findings were not considered to be the cause of death. All tissues were fixed in 10% (V/V) buffered formalin for standard period of time (3–6 days). Sections (5 mm thick) were cut from each block of paraffin-embedded tissues and were stained with haematoxylin and eosin (HE). Samples were also used for identifying any degenerative or inflammatory changes present.

Microbiological studies

Pharyngeal and nasal secretions were obtained by pharyngeal swabs and cerebrospinal fluid by puncture of the fourth ventricle. The swabs were taken and placed into transport culture medium at the scene within two hours of death was discovered. The parent(s) signed the informed consent and they got detailed information about the purpose of our investigation in SIDS cases. Heart-blood and lung tissues were obtained during autopsy. Virus antigens were detected in paraffin-embedded lung tissue.

Bacteriological methods

The specimens were streaked on the surface of blood and chocolate agar, incubated at 37 °C aerobically, anaerobically and at 5% CO₂ atmosphere. Plates were examined after 24 hours and 48 hours. The strains were identified by Cowan-Steel methods [3], Gram-negative rods were identified by bio-Merieux ID32E system or ID 32 GN system.

Yeast cultural methods

Sabouraud agar containing 10 mg/ml gentamicin was used for the isolation of yeasts. Isolates were identified by bio-Merieux ID32C system.

Immunohistologic procedures for detection of viral antigenes

Paraffin-embedded sections were dewaxed in xylene and immersed in 0.1% trypsin in PBS to release the overfixed antigenic sites. The sections were treated with 1% H₂O₂ in methanol. These sections were incubated with appropriately diluted monoclonal antibodies against the following viruses: Respiratory Syncytial viruses (Serotypes A, B), Influenza virus A, Influenza virus B, Parainfluenza viruses (Type 1, 2, 3), Adenoviruses (ARGENE, Biosoft, France). After incubation (2 h, 37 °C) bound monoclonal antibodies were detected with peroxidase labelled anti-mouse IgG (Sigma). Peroxidase activity was visualized using 3-Amino-9-Ethylcarbazole (AEC) substrate and sections were slightly counterstained with hemalum.

Detailed microbiological examinations of S. aureus strains

Isolates from the specimens of SIDS and healthy infants were screened for production of enterotoxin A (SEA), B (SEB), C (SEC), D (SED) and TSST-1 by Oxoid Toxin Detection Kit TD 940 and TD 900 (Unipath Limited, Hampshire, England). The kit may be used to detect staphylococcal entero- and TSST-1 toxins and to measure

semi-quantitative results in the culture filtrates of isolates by reversed passive latex agglutination.

Antibiotic sensitivity of *S. aureus* strains were tested on Müller–Hinton agar by penicillin, oxacillin, ampicillin, amoxicillin + clavulanic-acid, cefuroxim, erythromycin, clindamycin, roxithromycin, vancomycin, ofloxacin, ciprofloxacin, trimethoprim, gentamicin and nethilmycin disks (Oxoid discs). Methicillin resistance of *S. aureus* (MRSA) was detected on Müller–Hinton agar containing 6 µg/ml oxacillin and 4% NaCl incubated at 35 °C.

Phage typing of *S. aureus* was carried out as described by Blair and Williams [4].

ORs with 95% confidence intervals were used to evaluate differences in the frequency of bacteria and toxin production in the various categories.

Table I

Relevant clinical details of 13 SIDS cases

No.	Sex	Age	Anamnestic data	Birth weight (g)	Time of death	Site of death
Case 1	F	2 months	mild cold	2600	5.00	home
Case 2	F	6 months	mild cold, cysta colli with <i>Staphylococcus aureus</i>	3200	16.00	home
Case 3	F	4 months	mild cold	2800	3.00	home
Case 4	M	4 months	negative	2620	5.30	home
Case 5	M	2 months	mild cold	3250	11.40	home
Case 6	M	3 months	negative	2770	10.00	home
Case 7	M	2 months	negative	3300	6.00	home
Case 8	M	11 months	high fever without medical treatment in 6 months of age	2800	8.00	home
Case 9	M	10 months	high fever in 3 months of age	2950	0.30	home
Case 10	M	6 months	anus atresia at birth, cold, high fever for 3 weeks before death, resuscitation	2700	5.10	hospital
Case 11	M	5 months	negative	3000	23.25	ambulance
Case 12	F	2 months	pneumonia in 2 weeks of age	3100	16.32	hospital
Case 13	M	5 months	soor oris	3200	7.00	home

Results

Thirteen SIDS cases, 4 female and 9 male infants, were under one year of age, the majority were under the age of three months. They were found dead at their homes, and only 2 infant died in hospital. In most of the cases the time of death was the early morning hours (from midnight to 8 a.m.). Four infants had showed symptoms of mild cold before death and 3 infants had high fever, 5 months (case 8), 7 months (case 9) and 3 weeks (case 10) before death. In 8 cases the infants were lower than the average Hungarian birth weight (3,100 g). SIDS cases were found in spring, autumn and winter months. In one of the cases a twin with low birth weight was the victim of SIDS (Table I).

Autopsy and histology. Intrathoracic, subpleural, subepicardial and subcapsular thymic petechiae were found in SIDS cases. Subconjunctival petechiae were not detected in any SIDS case. Mild or severe pulmonary edema was found in most of the cases. Lymphoid cells or macrophages in the upper respiratory tract were detected in about half of the cases.

Microbiology. The following microbes were isolated from the autopsy specimen.

Bacteriology: In 2 cases *S. aureus*, in 6 cases different *Enterobacteriaceae* species, in 4 cases *S. aureus* and *Enterobacteriaceae* strains and in 1 case *S. aureus* and *S. pyogenes* were isolated. In case 1. *E. coli* 0:8 H:999, a non-enteropathogen serotype, was isolated from blood, cerebrospinal fluid and throat specimen of a newborn. The results of bacteriological cultures are shown in Table II. Yeast isolates were *Candida albicans*. High number of *C. albicans* was cultured from pharyngeal secretions in case 1, from lung tissue in case 3, from pharyngeal secretions and lung tissue in case 5, and from pharyngeal specimen of case 13 (Table II).

Virus antigens were detected: In case 7. *Parainfluenza Type 2 virus* antigen with previous negative clinical symptoms and very mild pathological changes. All other SIDS cases were negative (Table II).

S. aureus was the predominant isolate from specimens of SIDS cases, 7/13 (53%) (Table III).

SEA, SEB and/or TSST-1 toxins were identified only in 6 SIDS cases 6/13 (46%) (Table IV). Different amounts of SEA, SEB and TSST-1 were produced by these staphylococcus strains. Large quantities of SEA were produced by *S. aureus* isolated from Case 1. At the same time two or three different types of pyrogenic and TSST-1 toxins were produced by these *S. aureus* strains (Table V).

Among the 9 infants who died of other cause, different pathogens or normal microbial flora were isolated; however *S. aureus* was not cultured.

Table II

Results of microbiological examination of 13 SIDS cases

No.	Throat specimen	Pulmonary tissue	Blood culture	Cerebrospinal fluid
Case 1	<i>Staphylococcus aureus</i> <i>Escherichia coli</i> <i>Candida albicans</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Escherichia coli</i>
Case 2	<i>Staphylococcus aureus</i>	<i>Staphylococcus aureus</i>	<i>Staphylococcus aureus</i>	sterile
Case 3	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i> <i>Candida albicans</i>	sterile	sterile
Case 4	<i>Staphylococcus aureus</i> <i>Serratia rubidea</i>	<i>Staphylococcus aureus</i> <i>Serratia rubidea</i>	sterile	sterile
Case 5	<i>Staphylococcus aureus</i> <i>Klebsiella oxytoca</i> <i>Candida albicans</i>	<i>Staphylococcus aureus</i> <i>Klebsiella oxytoca</i> <i>Candida albicans</i>	sterile	sterile
Case 6	<i>Enterobacter aerogenes</i>	<i>Enterobacter aerogenes</i> <i>Enterococcus faecalis</i>	<i>Enterococcus faecalis</i>	sterile
Case 7	<i>Escherichia coli</i> <i>Enterobacter aerogenes</i>	<i>Escherichia coli</i> <i>Enterobacter aerogenes</i> <i>Parainfluenza virus</i>	sterile	sterile
Case 8	<i>Escherichia coli</i>	<i>Escherichia coli</i>	sterile	sterile
Case 9	<i>Staphylococcus aureus</i>	<i>Staphylococcus aureus</i>	sterile	sterile
Case 10	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i> <i>Enterococcus faecalis</i>	<i>Enterococcus faecalis</i>	<i>Enterococcus faecalis</i>
Case 11	<i>E. chloaceae</i>	<i>E. chloaceae</i>	sterile	sterile
Case 12	<i>S. aureus</i> <i>S. pyogenes</i>	<i>S. pyogenes</i>	sterile	sterile
Case 13	<i>E. coli</i> <i>C. albicans</i>	sterile	sterile	sterile

The screening of nasopharyngeal flora of 100 healthy infants under the age of one year showed normal microbial flora with only few number of *S. aureus* (37%). (Table III). There were only 16/100 (16%) toxin producers (Table IV). SEA, SEB, SEC, SED and/or TSST-1 toxins were detected (Table VI). Compared to the SIDS infants, the proportion of carriers and the quantity of the excreted toxins proved fewer numbers among healthy infants (Table VI). Distribution of toxigenic and non-toxigenic strains in *S. aureus* carriers were 85% pyrogen toxin producers in SIDS cases but 43% in healthy infants (Table VII).

Table III*Distribution of S. aureus strains in the investigated cases*

	<i>S. aureus</i>		Total
	positive (%)	negative (%)	
SIDS infants	7 (54)	6 (46)	13
Healthy infants	37 (37)	63 (63)	100
Total	44	69	113

OR=1.986 95% CI=0.55–7.33

Table IV*Distribution of toxigenic S. aureus in the investigated cases*

	Toxigenic <i>S. aureus</i> carriers (%)	Non-toxigenic <i>S. aureus</i> carriers and non- <i>S.</i> <i>aureus</i> carriers (%)	Total
SIDS infants	6 (46)	7 (54)	13
Healthy infants	16 (16)	84 (84)	100
Total	22	91	113

OR=4.500 95% CI=1.15–17.72

Antibiotic sensitivity of SIDS isolates: 6 *S. aureus* strains were resistant to penicillin and 1 strain to oxacillin; they were sensitive to other antibiotics tested.

The III phage group was dominant among *S. aureus* strains isolated from SIDS infants (Table V).

Discussion

During the first six months, infants undergo many physiological adaptations including the decrease of maternal antibody level and gradual induction of the infant's immune system following the influence of environmental antigens and immunization [5, 6]. In this period the normal microbial flora of epithelial and mucosal surfaces are

developed and this is a highly important factor contributing to the development of immune response [7, 8].

The microbial flora of infants who died in SIDS was studied. The role of isolates was assessed in the cases in which the same species were cultured from various organs or pathogenic bacteria and yeasts were isolated in large number. *S. aureus* and different *Enterobacteriaceae* strains were isolated alone or together most frequently from the pharynx and lungs of SIDS victims. *C. albicans* was also a frequently isolated microorganism.

Table V

Examination of staphylococcal strains isolated from SIDS cases

No. of patients/ Strains	<i>Staphylococcus</i> species	Enterotoxin*				TSST-1*	phag group	Localisation of isolation
		A	B	C	D			
1/1	<i>S. haemolyticus</i>	—	—	—	—	—		nose
1/2	<i>S. aureus</i>	1/64	—	—	—	—	III.	throat
2/3	<i>S. aureus</i>	1/8	1/8	—	—	1/4	mixed	throat
2/4	<i>S. aureus</i>	1/2	—	—	—	1/2	mixed	lung
2/5	<i>S. aureus</i>	1/2	—	—	—	1/2	mixed	blood
2/6	<i>S. haemolyticus</i>	1/1	1/1	—	—	1/2		lung
3/7	<i>S. epidermidis</i>	—	—	—	—	—		nose
3/8	<i>S. aureus</i>	1/32	1/32	—	—	1/4	III.	lung
3/9	<i>S. epidermidis</i>	—	—	—	—	—		nose
4/10	<i>S. aureus</i>	1/32	—	—	—	1/4	III.	lung
4/11	<i>S. aureus</i>	1/16	1/4	—	—	1/2	II.	throat
5/12	<i>S. aureus</i>	1/32	—	—	—	1/2	III.	throat
5/13	<i>S. aureus</i>	1/32	1/4	—	—	1/2	III.	lung
5/14	<i>S. haemolyticus</i>	—	—	—	—	—		nose
5/15	<i>S. haemolyticus</i>	—	—	—	—	—		nose
9/16	<i>S. aureus</i>	1/32	—	—	—	—	III.	lung
13/17	<i>S. aureus</i>	—	—	—	—	—	III.	throat

*Dilution ratio of the culture filtrates, where the endpoint of the reaction is

Our results agree with reports on the isolation of *Enterobacteriaceae* strains and *S. aureus* from SIDS infants [9–11]. These microorganisms are transient in low number in the upper respiratory tract, though the large number of these toxin-producing strains could be a decisive factor triggering the events leading to SIDS [12].

Table VI

Examination of staphylococcal strains isolated from the nasopharynx of healthy infants

No of patients/ strain	<i>Staphylococcus</i> species	Enterotoxin*				TSST-1*	phag group
		A	B	C	D		
101/a	<i>S. aureus</i>	1/2	1/2	—	—	1/16	II.
109/a	<i>S. aureus</i>	1/8	—	—	—	—	mixed
123/a	<i>S. aureus</i>	1/2	1/2	1/2	1/2	1/2	II.
123/b	<i>S. aureus</i>	—	—	—	—	—	II.
504/a	<i>S. aureus</i>	1/8	1/2	—	—	—	II.
505/a	<i>S. aureus</i>	1/2	—	—	—	—	II.
510/a	<i>S. aureus</i>	—	1/8	—	—	—	mixed
516/a	<i>S. aureus</i>	—	1/8	—	—	—	II.
524/a	<i>S. aureus</i>	1/1	—	—	—	—	III.
526/a	<i>S. aureus</i>	—	—	—	1/8	1/8	II.
601/a	<i>S. aureus</i>	—	1/8	—	—	—	II.
606/a	<i>S. aureus</i>	—	1/8	—	—	—	II.
704/a	<i>S. aureus</i>	—	1/4	—	—	—	III.
710/a	<i>S. aureus</i>	1/4	1/4	—	—	—	I.
714/a	<i>S. aureus</i>	1/8	—	—	—	—	III.
714/b	<i>S. aureus</i>	—	—	—	—	—	I.
717/a	<i>S. aureus</i>	1/2	—	—	—	—	III.
725/a	<i>S. aureus</i>	1/4	—	—	1/4	1/4	I.

*Dilution ratio of the culture filtrates, where the endpoint of the reaction is

a = throat isolate, b = nasal isolate

S. aureus strain plays a significant role in the pathomechanisms of SIDS. Many infants (40–50%) are colonized by *S. aureus* in the first few months of life [13, 14]. Pyrogenic toxins are produced only between 37–40 °C [15]. Normally the temperature of mucous membranes of respiratory tract is under 37 °C, therefore, increases in local nasal temperature as was observed in the prone position as respiratory discharge provide a favorable microenvironments for the production of toxin [16, 17]. The superantigen activity of pyrogenic toxins of *S. aureus* might trigger powerful inflammatory mediators [2, 18]. The significance of pyrogenic toxin-producing strains of *S. aureus* in the pathogenetic reactions of SIDS has been suggested by several researchers [5, 19, 20].

SIDS victims had a higher carriage rate of toxin producing staphylococci and coliforms in the nasopharynx than healthy infants in England [14, 21]. *S. aureus*, *Streptococcus* spp., *Enterococcus* spp., toxigenic *E. coli* in the nasopharyngeal flora and *C. perfringens* in faecal samples were present in higher numbers in SIDS infants

compared with infants who died of other causes and live infants in Australia [22]. *S. aureus* was found in 86% of Scottish SIDS but only in 56% of healthy infants [14].

S. aureus was isolated in high numbers of 2–4 month old infants. In this age group the peak of SIDS incidence was probably due to expression of the Lewis antigen, an epithelial receptor of *S. aureus*, found in nearly 90% of the infants. It has been suggested that the colonization of infants by *S. aureus* was growing in this age range [23].

Table VII

Distribution of toxigenic and non-toxigenic strains in S. aureus carriers

	Toxigenic <i>S. aureus</i> (%)	Non-toxigenic <i>S. aureus</i> (%)	Total
SIDS infants	6 (86)	1 (14)	7
Healthy infants	16 (43)	21 (57)	37
Total	22	22	44

OR=7.875 95% CI=0.78–191.89

In Hungary *S. aureus* was the predominant isolate from the SIDS cases. *S. aureus* was isolated from 54% of SIDS cases and only 37% from healthy infants /OR=1.986 (95% CI=0.55–7.33), $p=0.423$ /. According to our study toxigenic *S. aureus* was detected in 46% of SIDS cases and only 16% in healthy infants /OR=4.500 (95% CI=1.15–17.72), $p=0.010$ /. Considering all cases, the prevalence of *S. aureus* was twice higher and the toxin production activity of these strains was four and half times higher in SIDS cases than among healthy infants.

Distribution of toxigenic and non-toxigenic isolates showed a significant difference between SIDS (86%), and healthy infants (43%) /OR=7.875 (95% CI=0.78–191.89), $p=0.041$ /. This result shows higher possibility for carrying of toxigenic *S. aureus* in SIDS cases than in controls.

There was also a great difference in the characteristics of the toxin producing activity of our isolates. Two or three different types of pyrogenic toxins were detected in larger amounts in the isolates from SIDS cases than in the isolates from healthy infants. The superantigenic properties of these pyrogen toxins exert a markable effect on the immune system and formation of larger amount of cytokines.

In summary: We could detect significantly larger number of toxigenic *S. aureus* strains, larger amount of toxins, different types of pyrogenic toxin producing isolates in the SIDS cases than in the control group.

SEA and SEB producing isolates were predominant. Similar results were reported by a Hungarian pediatric study dealing with urticaria like toxic allergic exanthema in early childhood and upper respiratory tract infections caused by toxin producing *S. aureus* [24]. In our study 46% of SIDS victims carried toxigenic *S. aureus* strains. These results were similar to the ELISA and/or flow cytometry data of tissue and serum of SIDS cases reported by Zorgani et al [25]. They detected pyrogenic toxins in samples from SIDS infants in Scotland (56%), France (55%) and Australia (53%) [25]. Detection of pyrogen toxins in half of SIDS cases in these three countries and isolation of toxigenic *S. aureus* in nearly 50% of Hungarian SIDS cases suggest that these findings are not local phenomena.

Results of autopsy and histological examinations did not show any special features. The circulatory failure or inflammatory mediators, pyrogen endotoxins directly could cause petechial haemorrhages in the region above the diaphragma. Howat's pulmonary immunopathological examinations of SIDS cases have indicated similar results [7].

Superantigenic toxins of *S. aureus* and LPS of Gram-negative bacteria may be linked to antigens CD14 of alveolar macrophages and monocytes. Through TNF α induction they can cause fever and release of various inflammatory mediators, including IL1, interferon- γ and thrombocyte activating factors [26, 27]. The cytokines may cause heat or vascular shocks, hypoglycemia, deep sleep and apnoe leading to sudden death during the period of adaptation to life [14, 28].

S. aureus strains appear to play a significant role in the pathogenesis of SIDS. Therefore, microbiological screening for the newborns at SIDS risk is considered to be highly useful to find a factor of the multicausal SIDS.

Abbreviations: **SIDS** = Sudden Infant Death Syndrome, ***S. aureus*** = *Staphylococcus aureus*, **SEA** = *Staphylococcus Enterotoxin A*, **SEB** = *Staphylococcus Enterotoxin B*, **SEC** = *Staphylococcus Enterotoxin C*, **SED** = *Staphylococcus Enterotoxin D*, **TSST-1**=Toxic Shock Syndrome Toxin – 1, **OR** = Odds ratio, **CI** = confidence interval

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ALTERNATE ADENOVIRUS TYPE-PAIRS FOR A POSSIBLE CIRCUMVENTION OF HOST IMMUNE RESPONSE TO RECOMBINANT ADENOVIRUS VECTORS

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(Received: 21 March 2000; accepted: 5 April 2000)

With the help of monoclonal antibodies the existence of at least 18 different earlier not known intertype (IT) specific epitopes were demonstrated in different numbers and combinations on the hexons of different adenovirus serotypes. The IT specific epitopes play an important role in the experimental gene therapy and in the recombinant adenovirus vaccination because of the harmful immune response of the recipient organisms directed against the many different epitopes of the adenovirus vector. For the elimination of harmful effect the authors suggest the use of multiple vectors, each prepared from different adenovirus serotypes showing the loosest antigenic relationship to each other. The vectors would be used sequentially when second or multiple administration is needed. For this purpose the authors determined and described 31 such adenovirus type-pairs, which are probably the best alternates for sequential use in experimental gene therapy.

Keywords: adenovirus, antigenic relationship, recombinant adenovirus vector

Recombinant adenoviruses (rAdV) have been widely used as gene delivery vectors to target cells in experiments both with curative and preventive purposes. Adenovirus vectors have been used in the experimental gene therapy of genetic disorders, in immuno- and molecular therapy of a variety of cancers. Recombinant adenoviruses appear to be attractive candidates for vaccination against the infectious diseases, too. However, one of the major obstacles to their use is the strong cellular and humoral immune response of the host to the vector proteins. Neutralizing and non-neutralizing anti-vector antibodies may substantially inhibit the effect of recombinant adenoviruses especially when multiple administrations are required [1–4]. The explanation could be based on the complex antigenic structure of the hexon, the major coat protein of adenovirus, on which besides the genus- and type specific epitopes the

existence of numerous different intertype (IT) specific epitopes were demonstrated with monoclonal antibodies [5], and with prediction methods. The antigenicity and immunogenicity of synthesized peptides representing potential IT specific epitopes were also demonstrated [6]. It can be supposed that these IT specific epitopes play a significant role in the immune reactions both *in vitro* and *in vivo*. This prompted us to analyse the data concerning the epitope structure of the hexon and to determine the type-pairs showing the loosest antigenic relationships between each other.

Table I

31 alternate adenovirus type-pairs to develop recombinant vectors for sequential use in gene therapy

Adenovirus types	4,19 cluster I	8,9,9/13,10 cluster II	1,2,5,6 cluster III	7	12	35	13	26	41	18	27	SadV	16	BadV	2
12			6/11				5/10								
27				3/15	3/10	4/8									
35			5/13				5/10								
41		6/11		5/12	4/9	4/9									
BAdV2		6/11	5/11	3/15	3/10	4/8		4/10	4/7						
BadV3	1/16	1/15	0/15	1/13	1/8	1/8	0/10	1/12	0/9	1/10	0/8	1/11		1/6	

The epitope content of each two hexon types are shown at meeting points: first number represents the number of identical and the second one the number of the different epitopes

Methods

For the investigation of epitope composition of different adenovirus hexon types 61 mouse ascitic fluids containing monoclonal antibodies (MAbs) have been used. MAbs have been developed in three panels directed against purified hexons of human adenovirus (HAdV) types 1, 35 and bovine adenovirus (BAdV) type 2, respectively. The distinction and marking of the different epitopes recognized by the MAbs have been carried out by the determination of the composite cross-reactivity pattern, the titer in ELISA and the correlation coefficient of all the 61 MAbs with 21 different hexon types representing all the six human subgenera, as well as different bovine and simian adenoviruses (SAdV) (see on Table I, upper horizontal row). The IT specific epitopes

defined by MAbs have been characterized by two groups of adenovirus serotypes: on which they are and on which they are not present [6]. The antigenic structure of the individual hexon types have been characterized by the determination of their IT specific epitope spectrum. The degree of antigenic relationship in the pairwise analysis has been expressed by the number of the identical and different epitopes present on any two given hexon types [5].

Results and discussion

The three panels of MAbs have recognized 22 different epitopes on the 21 hexon types among them a genus and three type specific ones and 18 different bi- and multilateral IT specific epitopes that grouped adenoviruses within the genus, independently from the subgenus they belong to.

The distribution of the distinct IT specific epitopes on the different hexon types has been different and varied between 1 and 17. By pairwise analysis ten human hexon types have formed three epitope clusters showing identical epitope spectra, namely type 4 and 19 (cluster I), types 8, 9, 9/13 and 10 (cluster II), as well as types 1, 2, 5 and 6 (cluster III) containing 17, 16 and 14 identical epitopes, respectively and no different epitopes at all. Close antigenic relationship was demonstrated between the types of the three different epitope clusters, each two given types containing 13 to 16 identical and only 1 to 4 different IT specific epitopes. HAdV types 7, 3, 18, 26 and SAdV type 16 also display a closer or looser but significant antigenic relationship among each other and to the members of the three clusters. HAdV serotypes 12, 27, 35, 41 and bovine adenovirus types 2 and 3, however, have shown a loose antigenic relationship with at least two or more other adenovirus types. Adenovirus type-pairs having the loosest antigenic relationships are shown on Table I. The number of identical epitopes on the given two hexon types varies between 0 to 6, and that of different epitopes on any given type-pairs are nearly double or manifold than that of the identical epitopes. Each two HAdV and/or BAdV serotypes determined by the meeting points are probably the best alternates for sequential use in experimental gene therapy.

On the basis of the present data a total of 31 different adenovirus type-pairs or small groups of types could be chosen for preparation of second or multiple recombinant vectors. For example in case of HAdV type 5, which is the most frequently used vector worldwide in the experimental gene therapy, for the second or multiple administration a recombinant vector could be used prepared from (i) bovine adenovirus type 3 which has no identical IT specific epitope with type 5, although the number of the different epitopes on the two types is 15, (ii) human adenovirus type 35

which has 5 identical epitopes, but the two types contain 13 different epitopes, (iii) bovine adenovirus type 2 having 5 identical epitopes and the two types contain 11 different epitopes, (iiii) human adenovirus type 12 having 6 identical epitopes with type 5, and the two types contain 11 different epitopes on their hexons.

Immune response of the host is induced not only by the genus and the type specific but also by the several IT specific epitopes, which have been found in different numbers and combinations on the different adenovirus serotypes. If well-chosen different serotypes are used as vectors for the first and second multiple administration the harmful effect of antibodies induced by the numerous IT specific epitopes could be eliminated. On the base of these data authors suggest the use of multiple vectors in gene therapy, each prepared from different adenovirus serotypes. The vectors would be used sequentially when second or multiple administration is needed.

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GROUP-F STREPTOCOCCAL PLEURO-PERICARDITIS IN A MESOTHELIOMA PATIENT AFTER DENTAL SURGERY

(CASE REPORT)

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(Received: 1 June 2000; accepted: 11 September 2000)

A 71-year-old mesothelioma patient developed pleuro-pericarditis and pleural empyema. Bacteriological examinations and serological identification proved group F *Streptococcus* in the pleural fluid. Anamnestic data suggested that the source of infection might have been the oral cavity after dental surgery.

Keywords: group F *Streptococcus* infection, mesothelioma

There are many species and serogroups of Streptococci. According to Lancefield classification Group-F streptococci represent a very diverse group. *Streptococcus anginosus*, *S. intermedius*, *S. constellatus* can be classified as viridans streptococci or *S. milleri*-group and all bacteria of these species can belong to F group of Streptococci. They show β or α haemolysis and normally are present in the oral cavity, the gastrointestinal tract and the female genital tract. They can be responsible for pyogenic infections, purulent suppurative lesions such as pleural empyema, meningitis, endocarditis, brain, liver, abdominal and subphrenic abscesses [1–7].

Here the authors summarize a case about a mesothelioma patient who developed pleuro-pericarditis caused by group F *Streptococcus* after dental surgery.

* Corresponding author

Materials and methods

Bacteriological examinations were performed from pleural aspirate gained by thoracoscopy. The cultivation was performed on bovine blood and chocolate agar. Based on macroscopic colony morphology and catalase test, serological identification was performed with OXOID's Streptococcal Grouping Kit. Antibiotic susceptibility testing was done on Mueller-Hinton blood agar with antibiotic paper discs of HUMAN, OXOID and Becton-Dickinson.

Results and discussion

A 71-year-old woman was admitted to the Intensive Care Unit of the Pulmonology Clinic of the Semmelweis University of Medicine (Budapest, Hungary) for thoracoscopy. The indication of this intervention was her recurrent pleural and pericardial effusion. After thoracoscopy she developed fever – that is not unusual after such intervention –, the aspirate of the pleural fluid was thick, viscous and turbid. Bacteriological examinations of all the three consecutive pleural aspirates (native and cultured in blood culture bottles) resulted in a peculiar β -haemolytic *Streptococcus* on bovine blood agar and α haemolytic on chocolate agar plates in pure cultures.

Each isolate coagglutinated in group F streptococcal antibody solution and was sensitive to a variety of penicillins, cephalosporins, macrolides and glycopeptides tested. Meanwhile the patient was treated with amoxicillin + clavulanic acid and her pericardial effusion ceased, her pericarditis and pleural empyema healed (Figure 1). However, her hydrothorax has recurred. The histological examination of the excindate by thoracoscopy has finally proved mesothelioma.

Serial microbiological examinations were performed from pleural punctates and drains in order to check the possible infectious origin of the recurrent hydrothorax. All the cultures were negative and the histopathological result proved our supposal that the recurrence of pleural effusion was due to the malignancy.

Anamnestic data suggested that the source of group F Streptococci might be the oral cavity: eight of the patient teeth were extracted prior to the admission for thoracoscopy. We have checked samples of both oral cavity and nose and throat for a possible group F streptococcal carrier state, but these cultures have been negative for group F *Streptococcus*, attributable to the prior appropriate antibiotic therapy. On the basis of the results of microbiological and histological examinations we have considered the pleural empyema and pericardial effusion to be of infectious origin in our patient at the time of admission. The subsequent recrudescence bacteriologically

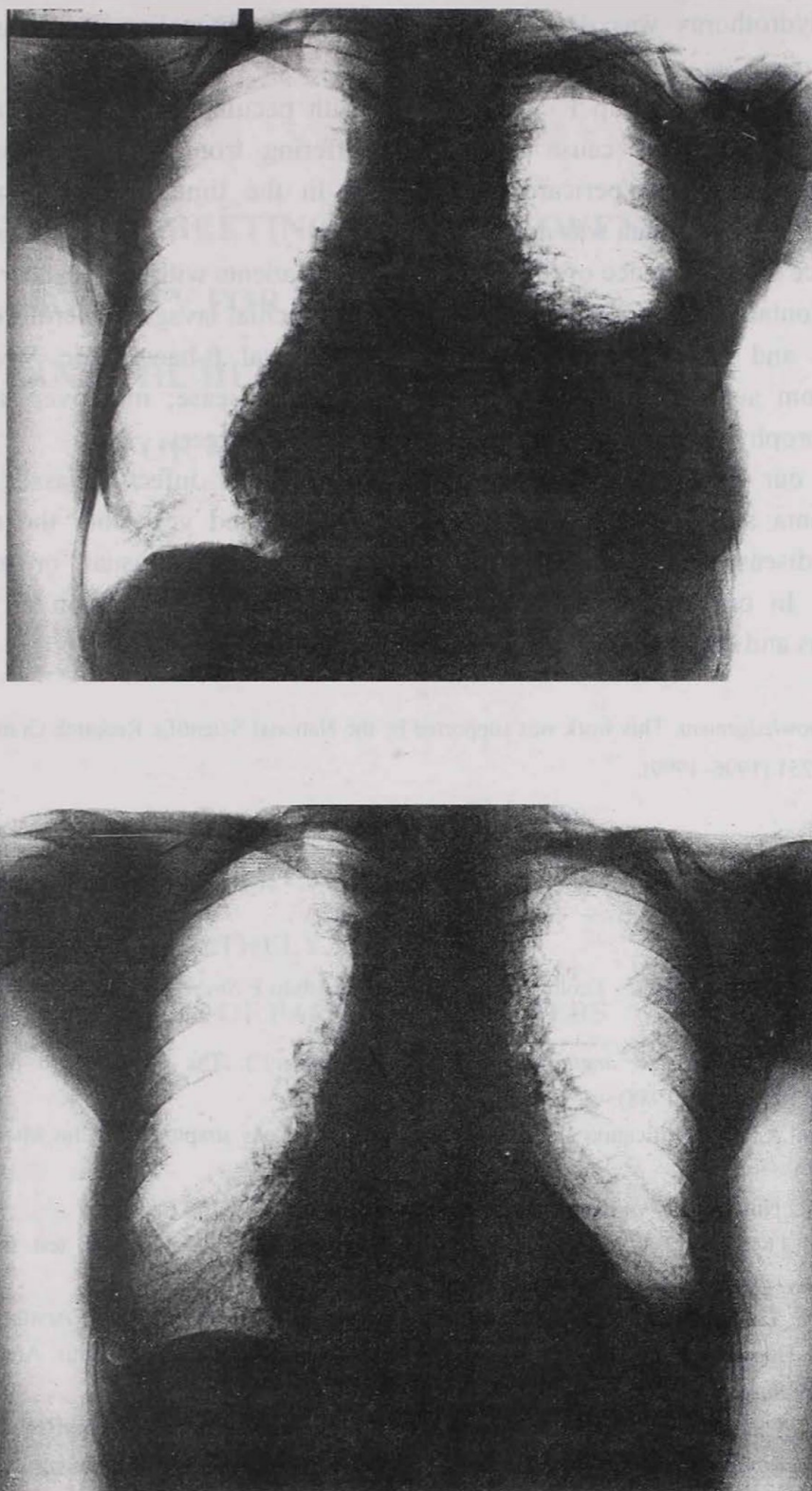


Fig. 1. Chest X-ray before and after treatment

negative hydrothorax was due to the metastasis of the malignant disease without infection.

In conclusion, group F *Streptococcus* with peculiar β -haemolytic activity on bovine blood agar may cause in patients suffering from malignant disease life-threatening pleural and pericardial infections. In the time of dental surgery the diagnosis of mesothelioma was not established yet.

Since the occurrence of this case, two other patients with lung cancer have been shown to contain group F *Streptococcus* in their bronchial lavages. Therefore, bacterial cultivation and serogrouping examination of unusual β -haemolytic *Streptococcus* isolates from such patients with known malignant disease; moreover, appropriate antibiotic prophylaxes are recommended prior to dental surgery.

To our best knowledge group F *Streptococcus* infection associated with mesothelioma and dental surgery has not been reported yet. Both the number of malignant diseases and the number of infections caused by "unusual" or rare bacteria increasing. In our case report we would like to draw the attention on such rare associations and emphasize their importance.

Acknowledgement. This work was supported by the National Scientific Research Grant of Hungary OTKA T 021251 (1996–1999).

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**FIRST JOINT MEETING OF THE SLOVENIAN
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ABSTRACTS OF PAPERS AND POSTERS

MYCOLOGY AND INDUSTRIAL MICROBIOLOGY

ZS. ANTAL¹, L. MANCZINGER², L. KREDICS², L. FERENCZY^{1,2}

Physical and functional investigation of the pThr1 plasmid

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Trichoderma species are common soil-born fungi. These species are economically important, in part because of their mycoparasitic ability, which makes them suitable for application as biocontrol agents against soil-born plant-pathogenic fungi. Despite the importance of these fungi, little is known about their extrachromosomal genetic elements.

In addition to the high-molecular-weight band, two lower-molecular-weight bands, 1.5 and 3.0 kb in size, were observed in the agarose gel electrophoresis patterns of undigested mtDNA preparations purified from the isolated mitochondria of a mycoparasitic *Trichoderma harzianum* strain. This pattern was produced by single molecules, 2.6 kb in size, that were supercoiled to various degrees. There were no sequence homologies between this plasmid, called by us pThr1, and the mtDNA. The complete 2622 bp sequence of the pThr1 plasmid was determined and analysed by means of computer programs. The plasmid displayed no homology at a DNA level with any mitochondrial plasmid or other DNA sequences accessible in the Genbank database. The sequence analysis revealed that the pThr1 plasmid contained three open reading frames (177, 150 and 1275 bp long) and the putative protein product of the long open reading frame, for a length of 650 bp, was similar to the reverse transcriptase of the Mauriceville and Varkud plasmids from *Neurospora* spp. Moreover, this open reading frame had conserved sequence elements, Q, R and S, characteristic of group I introns.

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K. ÁCS, ZS. KASZA, M. VASTAG, CS. VÁGVÖLGYI

Cloning and partial sequence analysis of the glyceraldehyde-3-phosphate gene of *Mucor circinelloides*

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The glyceraldehyde-3-phosphate gene (*gpd*) of *Mucor circinelloides* f. *lusitanicus* (ATCC 1216b) has been isolated from a genomic library by homologous hybridisation with a 350 bp fragment of the *gpd* gene amplified by PCR using specific primers. Until now about 70% of the entire *gpd* sequence has been identified. This sequence consists of 504 bp coding region from the 3' terminus and is not interrupted by introns. The amino-acid sequence encoded by the fragment shows a high degree of sequence identity with the corresponding gene products of various fungal species. Multiple alignments of fungal GPD sequences resulted in the construction of a phylogenetic tree. Results of the phylogenetic analysis of these sequences indicated a closer connection of the zygomycete *M. circinelloides* to the investigated basidiomycete than the ascomycete species. Sequence analysis of 307 bp of the 3' terminal non-coding region was also carried out and revealed the presence of fungal terminal consensus sequences.

This study was supported by OTKA grant T032738.

M. BATIČ, P. LIKAR, E. RUPNIK

Effect of C/N ratio on RNA accumulation in yeast

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During batch bioprocess the content of ribonucleic acid (RNA) in the yeasts varied depending on growth phase and medium composition. In yeast *S. cerevisiae* and *C. utilis* the effect of different C/N ratio (5:1, 10:1, 25:1 and 50:1) to RNA accumulation was studied in modified yeast malt extract broth medium at pH 4.0 and 6.0. During the bioprocess the pH, biomass concentration and RNA was followed. Results showed negative correlation between RNA content and increasing C/N ratio in the media. Cultivating *S. cerevisiae* and *C. utilis* 24 hours in YMB medium in side arm flasks at starting pH 4.0 and 6.0 at 28 °C on a rotary shaker with 200 rpm. The yeast biomass was followed by measurement of OD at 650 nm. The dry yeast biomass was

estimated by calibration curve prepared by filtration of the broth samples during growth. The RNA determination was performed in yeast biomass by extraction of RNA with 0.5 M HClO₄. Extracted RNA was centrifuged at 14,000 min⁻¹ and absorbency was measured at 260 nm in supernatant. Calibration curve prepared by the same procedure with RNA from *Torula* yeast was used for estimation of total yeast cell RNA of *S. cerevisiae* and *C. utilis*.

During the growth of *S. cerevisiae* and *C. utilis* in batch bioprocess the pH fall and biomass concentration increased with increasing concentration of carbon in the media. Contrary, nitrogen limitation caused reduction of maximal specific growth rate and RNA accumulation of both yeasts. Increased C/N ratio from 5:1 and 10:1 to 50:1 resulted in 38% to 20% reduction of RNA measured at the end of the bioprocess. Results showed that growth of yeast in YMB media with nitrogen limitation caused 20 to 40 minutes prolongation of cell doubling time (tg) at *S. cerevisiae*. In *C. utilis* the RNA concentration decreased from 36 mg RNA/g d.w. at C/N ratio 10:1 to 33, 34 and 26 mg RNA/g d.w. at C/N ratio 5:1, 25:1 and 50:1 in the cultivation media. The CIN ratio in growth medium of yeast *S. cerevisiae* and *C. utilis* plays important role in RNA accumulation of yeast cells. The negative effect on RNA accumulation in yeast cell appeared when C/N increased from 5:1 and 10:1 to 50:1. Contrary, biomass production increased up to 10.6 g/L when high nitrogen limitation media was used.

ZS. BENKŐ, M. MISKEI, CS. FENYVESVÖLGYI, V. BENESOCZKI, M. SIPICZKI

**Effect of mutations causing caffeine resistance on cell cycle
and their interaction with other mutations. Adaptation to caffeine**

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Caffeine is one of the most frequently consumed drugs. Several aspects of the effect of caffeine on living organisms are studied. Nascent resistance against medicines and various toxins cause serious problems for medical science. The latest results showed that caffeine could not cause cancer alone but it could increase effectively the carcinogen effect of several compounds. In addition the caffeine is a well-known toxin of cells with different effects on them.

To better understand the effects of caffeine we have isolated caffeine-resistant mutants that could tolerate 4-5 times higher concentration of caffeine than the wild type cells. One representative was chosen from the four-complementation groups for further studies. Analysis of these mutants revealed several dissimilarity to wild-type cells such as prolonged cell cycle, elongated cells, UV sensitivity, γ -resistance, pH-sensitive

growth, damaged meiosis, starvation sensitivity and brefeldin A resistance. Molecular analysis revealed that the *caf1* gene product is probably responsible for nuclear transport. The *caf2* gene encodes an exportin protein. The *caf3* gene product is an AP-1-type transcription factor. The *caf4* gene encodes thioredoxin reductase enzyme. These results suggest a very complex system existing in the cell that is able to reduce the toxic effect of caffeine.

Effect on cell cycle. It is known that hydroxy urea (HU) blocks the cells in the S phase. Addition of caffeine to the blocked wild-type cells can override the S-M checkpoint that result in cell death. We found that the HU-blocked *caf* mutants can tolerate the addition of caffeine. The most common changes in phenotype of *S. pombe* cells following addition of caffeine are the prolonged cell cycle. To understand this effect more clearly we isolated further caffeine-resistant mutants that have extremely prolonged cell cycle both with and without caffeine in the media.

Interaction with other mutation. The *caf3-89* dominant mutation (AP-1-like transcription factor) increases morphological changes in phenotype caused by *sepl* mutation while the *caf3-89 sepl3-572* double mutation is lethal. The *caf3* gene even interacts with some auxotroph mutations especially with *ade6*. We have isolated a sterile *ste*y mutant that causes reduced caffeine-resistance and altered cell morphology in the *caf3-89 ste*y double mutant.

Adaptation to caffeine. We also found that *S. pombe* cells could adapt to higher concentration of caffeine. This adaptation is reversible. It has turned out that the adaptation ability due to spontaneous mutation while the wild-type strains have reduced ability to adapt to caffeine. To know more about the mechanism of adaptation we have started to study both the wild-type and the mutant strain.

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Investigations on the vitality, resistance and diversity of metal-adapted and non-adapted *Rhizobium* strains

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Waste disposal of the sewage sludge in the arable lands is a common agricultural practice throughout the world. After the long-term applications, however, some diverse effect of the accumulated heavy metals can be detected on some soil-biological groups and parameters. Among them the bacterial and fungal (*Rhizobium*

and AMF) microsymbionts are sensitive enough to use them as potential, appropriate eco-toxicological biosensors (Balla et al. 1998).

Growth-rate and metal-tolerance of adapted and non-adapted *Rhizobium leguminosarum* bv. *trifolii* isolates were tested *in vitro*. Strains were originating from a heavily polluted soil, where various rates of communal sewage sludge were used regularly for more than ten years (in Hungary). Five concentrations (0.1, 1, 10, 100 and 1000 mg l⁻¹) of some heavy metal salts (Zn, Cu, Co and Ni, as sulphates and chlorides) were used in a micro-fermentor, where the cell number of the tested strains was estimated after 14 hours of incubation. Starting ratio of the rhizobium cells was set to 10⁶ CFU ml⁻¹ in liquid YEM, yeast-extract mannitol-media (inoculated by about 100 µl/tube after a Thoma-chamber counting and calculation). After 14 hours of growth (at 28 °C) in a rotary shaker, optical density (OD = 640 nm) of the suspensions were measured and compared to the metal-free control. Modified plate count method was also used to assess the abundance of the culturable cells (Angerer et al. 1998). Plasmide profile analysis (PPA), as phenotypic community characteristic, was used to indicate the microbial diversity of the rhizobia in the sewage sludge treated plots. Data were analysed statistically.

Adapted *Rhizobium* strains, which were selected after 10 years of regular sewage-sludge treatments, were found to be almost uniformly tolerant to all of the applied heavy metal rates. Strains, originating from an unpolluted soil, however, were generally sensitive to as low, as 10 mg.kg⁻¹ concentrations of the metals. The lowest doses of some microelements (at about 0.1 and 1 mg.kg⁻¹) stimulated the growth, depending on the metals and the strains, as it was found in an earlier study (Kecskés et al. 1997).

Cell number of adapted rhizobia was found to be less by about twofold compared the untreated and non-adapted controls. Vitality and growth of the metal-tolerant strains decreased by about 60% at all of the treatments after a 15-years of metal contamination. Maximal genetical- (plasmide profile) diversity was present at the non-polluted soils, whereas the communal sewage sludge application has decreased it as a function of the long-term effect, the soil-physical-chemical characteristics and the usage of the lands.

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A. BOZSIK^{1,2}, M. SIPICZKI^{1,2}

**The cytological and genetical study of the dimorphic yeast
*Schizosaccharomyces japonicus***

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S. japonicus is a unicellular haploid fission yeast. Life cycle has dimorphism: young colonies consist of cylindrical single cells and after 8–10 days on solid medium they develop hyphae characteristic of real fungi. We put emphasis on studying this dimorphism. We highlighted the differences between single cells and hyphae in cytological aspects: with respect of mitosis and the pattern of cytoskeletal elements the two forms shared the same morphology with a slight difference due to the shape of the cells. We generated auxotrophic mutants from the wild type strain.

We divided the isolated mutants into complementation groups; the exact spot of some of these groups were determined in the respective biosynthetic pathway. We performed protoplasts fusion. The segregation of alleles originating from azygotic asci turned to be mendelic. We isolated several hyphae-producing mutants and mutants that show sensitivity or resistance to hyphael growth-blocking toxins. Studying their genetical background we found important genes participating in metamorphosis. During the fluorescent paint of the nucleus we noticed three chromosomes in the cell. With pulsed field electrophoresis (CHEF) only two bands could be detected on the gele. Perhaps two chromosomes have the same size. Transformation was difficult: *S. japonicus* cannot apply the ARS of *S. pombe*. We generated *S. japonicus* library in *pJK148* integrative plasmid and searched for specific ARS. With the help of this gene we elaborated an effective transformation system for *S. japonicus*. We are able to transform metamorphosis mutants and get more details about the genes regulating this phenomenon.

A. BRÜCKNER, CS. BALLA, I. KISS

Effect of map on spoilage microorganism of cut vegetable

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In consequence of new consumer demands of the last decades fresh-like vegetable and fruit products with a shelf-life of few days appeared on the market.

Changes in quality – during modified atmosphere storage at 5 °C – of cut carrots and Chinese cabbage were examined. These quality changes were followed by the growth of spoilage microorganism populations (total counts of mesophil aerobe bacteria, *Pseudomonas*, lactic acid bacteria). Modified atmosphere in the sample packages was established by packaging into materials of different permeability to gases and by added CO₂ or O₂. So the atmosphere in the packages was normal (air), rich in CO₂ – poor in O₂ or rich in O₂. To analyse the growth curves of microorganism populations the Baranyi-model was used.

High CO₂ concentration restricted the growth of mesophil aerobe microorganism and *Pseudomonas* bacteria, but it had a beneficial effect on lactic acid bacteria, which were present in the starting microflora in more order lower numbers than mesophil aerobe microorganisms. High O₂ level had restrictive effect on mesophil aerobe microorganisms in case of Chinese cabbage. Growth of microorganism in carrot samples did not show the same trend.

Each product needs different gas composition to achieve beneficial influence on the quality of different vegetables. Beside microbiological changes also other factors like plant respiration, sensorial changes have to be taken into consideration in case of planning a new product or choosing a technology.

G. BUJDOSÓ¹, C. EGLI², T. HENICK-KLING²

**Fast method for the identification and differentiation of species
and strains belonging to genera *Hanseniaspora* *Kloeckera* by traditional
and molecular biological methods**

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The conversion of grape juice into wine is a complex process that is carried out by successive of various yeasts [1, 2]. The fermentation of must or juice is often initiated by the strains of *Hanseniaspora* *Kloeckera* predominantly what can cause lot of concerns to winemakers regarding the off-flavors, e.g., acetic acid, acetaldehyde, ethyl acetate, these yeasts produce during their proliferation [3, 4]. Others although consider them as positive determinants what can contribute to wine with characteristic flavors by producing acetoin, 2,3-butanediol, glycerol and other higher alcohols [5, 6]. To know more about their biology, it is important to establish an easy and fast method for the identification and differentiation of the species within the genera of

Hanseniasporal Kloeckera. Yeasts from different places around the world were examined to evolve an identification scheme for these genera. Physiological methods and molecular biological techniques, e.g., ITS-PCR, RAPD-PCR, Microsatellite-PCR and RFLP, were used to the assay. By the invented method strains belonging to these genera were identified and differentiated accurately. This procedure allows a fast and reliable methodology for the classification of strains of *Hanseniasporal Kloeckera*.

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G. CSITÁRI, G. KEREKES, K. LÁSZLÓ

Effect of zineb on microbial activity in different soils

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Zineb (zink-ethylene-1,2-bis-dithiocarbamate) is a dithiocarbamate type fungicid with broad-range specificity. It is commonly used as a foliar fungicid, but it is also allowed for partial soil sterilization in some culture. Effect of pesticides containing zineb as active ingredient on soil microbial activity was evaluated. The soils used in the experiments differed from each other in humus and clay content (1.15–6.35 percent humus, 28–55 soil plasticity index $/K_A/$). Measurements of microbial activity were based upon microbial biomass and three soil enzyme (dehydrogenase, phosphatase and invertase) activities. These enzymes differed from each other not only in substrate specificities but in their stability, too. Dehydrogenase activities bind to the intact, living cells, phosphatase activities maintain their activities after releasing from cells in the soil solution for weeks, invertase can be immobilised to soil colloids and remains active for years.

We concluded that soil type and fungicide treatment had significant effects on all variables measured (microbial biomass and enzyme activities) at 0.05 probability level. The correlations between the variables were determined by statistical methods. Correlation coefficients between the microbial biomass and enzyme activities were 0.435, 0.677 and 0.902 (dehydrogenase, phosphatase and invertase), respectively: The coefficients increased with the increasing stability of enzymes. There is a similar relationship between the microbial variables and soil properties (clay and humus content, pH and water holding capacity $/WHC/$): invertase activity and microbial

biomass showed the strongest, dehydrogenase activity showed the weakest correlations. The correlations were positive in case of humus percent, clay content and WHC, they were negative in case of pH.

The efficiency of fungicide treatments also changed in significant manner in different soils. The correlation between the soil properties and the efficiency, however, was weak.

K. CZAKÓ-VÉR, ZS. KOÓSZ, M. PESTI

**Classical genetic analysis of the *Schizosaccharomyces pombe*
chromium(VI)-sensitive and tolerant mutants**

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Hexavalent chromium(Cr(VI))-sensitive and tolerant mutants were isolated from *Schizosaccharomyces pombe* strains, with different auxotrophy and mating type. The following mutants were selected: wild-type mutant (CW-6, lys^- , h^+), a chromium sensitive (CS-6.51) and a tolerant one (CT-6.66) and other wild-type strain (CW-9, leu^- , h^-) and its tolerant mutant (CT-9.204). For the crosses two wild-type strains with uracil auxotrophy were used (CW-189, ura^- , h^+ and CW-190, ura^- , h^-).

The aim of this work was to determine and characterize the gene/genes involved in chromium tolerance and sensitivity. The classical genetical analysis was started with the ploidity test and mating type control, after the control of the spore-forming ability, tetrad analysis was done.

The sporoclones were tested for chromium tolerance and auxotrophy.

(i) CS-6.51 $\times$ CW-9 crosses: analysing 113 tetrads, only 17% of the sporoclones were viable, one complete tetrad were got, so for the determination of the genetic background this cross was not useful

(ii) CT-6.66 $\times$ CW-9: analysing 40 tetrads in 17 case were 2:2 segregation, 74 wild-type, 51 tolerant and 34 sensitive clones out of 160 clones were got

(iii) CT-6.66 $\times$ CW-190: 11 out of 27 tetrads were 2:2 segregation, out of 108 sporoclones 42 wild-type, 48 tolerant, 18 not detectable

(iv) CT-9.204 $\times$ CW-189 9 tetrads out of 15 were 2:2 segregation, the clones were all parental ditype

For the transformation 5 clones, with chromium tolerant phenotype and uracil auxotrophy were selected.

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Optimization of the green fluorescent protein (GFP) – blue fluorescent protein (BFP) dual tagging system in plants

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The green fluorescent protein (GFP) gene from *Aequorea victoria* has rapidly become of extensive use as a versatile vital marker in various organisms. Its fluorescence can be detected conveniently with non-invasive methods. Applications of fluorescent proteins have expanded greatly due to the availability of GFP mutants with altered spectral properties. One of these is the blue fluorescent protein (BFP) with an emission maximum shifted to shorter wavelength range. While GFP is routinely used in higher plants, there are only few data concerning the use of BFP. These proteins together are used for dual colour tagging experiments in other biological systems, however the benefits of this technique in plants have not been exploited yet.

In this study, our aim was to optimize the GFP-BFP double labelling method in plants. Such system has the capacity for use in virological experiments where the exact localisation of viral components and the kinetics of their expression could be studied. Distinct *Nicotiana* species were infected by plant virus vectors carrying the GFP or the Y66H type BFP gene. Fluorescence in the epidermal cells was detected with epifluorescence microscopy. While GFP expression level was high in every infected species, the level of BFP expression varied in wide range between the different *Nicotiana* species. Possible explanations for this phenomenon will also be discussed.

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Can complementary biological oxidation be applied for treatment of toxic acid tar waste? – A biodegradation test

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The development of the wastewater treatment is one of the most important steps in the course of the modernisation of the Hungarian Oil and Gas Co. (MOL Rt.) Danube Refinery. A lot of wastewater treatment methods should be introduced in order to decrease the level of solved organic materials in the extremely polluted outlet waters of the works. Our goal was to investigate the applicability of the complementary

biological oxidation that follows the chemical pretreatment of the highly acidic industrial wastewater.

The acid tar is a by-product of paraffin-refinement. It consists of sulphuric acid, sulphonated hydrocarbon fractions and carboxyl acid derivatives. The chemical oxygen demand (COD) of the fluid phase was 30000 mg/l. The chemical treatment was an oxidation with hydrogen peroxide followed by neutralisation. The COD of the fluid phase was reduced to 8800 mg/l and the total organic carbon (TOC) became 3500 mg/l.

Biological oxidation examination was performed from 2 and 10 times diluted samples of the pretreated water. The inoculum was activated sludge derived from the biological wastewater treatment system of the plant. The degree of biodegradation was determined by measuring COD and TOC.

On the basis of both COD and TOC data substantial differences were found in the extent of degradation by use of the two dilutions. A reduction of 360 mg/l COD (8%) was found for the 2 times diluted samples, and the reduction of 430 mg/l (47%) was found for 10 times diluted samples. The examination of the inhibitory action and the activity of the activated sludge demonstrate that the easily utilisable carbon and energy sources (glucose, acetate) are metabolised by the activated sludge at a high rate, however, their degradation decreased with an increase of a ratio of wastewater.

Processes in the degrading culture were also checked by the measurement of the inorganic carbon content (IC), too. The initial relatively homogenous IC level was reduced except in dishes containing higher amount of wastewater. It means that the possible biological oxidation had already been largely occurred. Provided that the COD is mainly caused by organic compounds the COD/TOC ratio could suggest the biological oxidation of the organic compounds. At the beginning of the experiment the ratio was usually higher than 24 hours later. One day later the biggest change in this ratio was in samples not containing wastewater. However, ratio was hardly reduced when wastewater was present in high volumes, moreover, a slight increase was sometimes found suggesting inhibitory action of wastewater on biological oxidation.

When the dilution was increased the ratios were reduced in greater extent meaning that the biological degrading process can be successful only with the application of at least 10× dilution. The wastewater system of the refinery possess substantial buffer capacity (even 100× dilution), thus we think that acid tar wastewater pretreated with chemical oxidation can reach a degree when its toxicity is eliminated.

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Effect of the phenoxyacetic acid, phenylacetic acid and their derivatives on the glutathione metabolism and antibiotic production of *Penicillium chrysogenum*

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Although penicillin G and V are among the first known antibiotics we still have, surprisingly few data on the metabolism of their side-chain precursors, phenylacetic acid (PA) and phenoxyacetic acid (POA) are available. In this work we studied the effect of PA, POA and their hydroxyl derivatives (2-hydroxy-PA, 3-hydroxy-PA, 4-hydroxy-PA, homogentisic acid, protocatechuic acid, 2-hydroxy-POA and 4-hydroxy-POA) on the glutathione (GSH) metabolism and penicillin production of an industrial strain of *Penicillium chrysogenum*. We also determined their minimal inhibitory concentrations (MIC) and their dissociation constants (pK). The following results were obtained:

1. PA and POA proved to be the most toxic compounds (MIC values 0.36% and 0.72, respectively). 2-Hydroxy-PA and 2-hydroxy-POA were also toxic (MIC values 0.74 and 0.92%, respectively) but all the others showed only negligible toxicity (MIC values higher than 1.8%).

2. Although these compounds are known to be transported into the cells by passive diffusion which depend on their acidity, we did not find any correlation between their toxicity (MIC) and their first dissociation constants. The pK₁ values of PA and all the PA derivatives were between 4.08 and 4.25 while in case of POA and its derivatives between 2.77 and 2.93.

3. The toxic compounds e.g. PA (0.25%), POA (0.5%) as well as 2-hydroxy-PA (0.7%) and 2-hydroxy-POA (0.7%) decreased the intracellular level of GSH which is an effective inhibitor of penicillin biosynthetic enzymes. They also induced both glutathione S-transferase and γ -glutamyl-transpeptidase, the first two enzymes of the GSH-dependent detoxification of xenobiotics. All the other compounds had no any effect on the GSH metabolism at 1% concentration.

4. Penicillin production did not correlate with either the toxicity of the potential side-chain precursors or their effect on the glutathione metabolism. The highest antibiotic productions were found in the presence of PA (3.8 mol/kg protein) and POA (3 mol/kg protein). Relatively high values were measured with 4-hydroxy-PA and 4-hydroxy-POA as well (0.9 and 1 mol/kg protein, respectively). With all the other compounds the penicillin productions were less than 0.5 mol/kg protein.

S. FARKAS, H.E.A.F. BAYOUMI HAMUDA, M. KECSKÉS

Inhibition of nodulation induced by *Rhizobium leguminosarum* bv. *phaseoli* on the root of bean (*Phaseolus vulgaris* L.) *in vivo*

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Inhibition of nodulation induced by *Rhizobium leguminosarum* bv. *phaseoli* strain was examined on the root hair of bean (*Phaseolus vulgaris* L.).

The seedlings were pretreated with promethazine or trimipramine in different concentrations. The effect of promethazine on the formation of bean root nodules and root hairs were examined *in vivo*. Promethazine was toxic for the seedlings at 40 µg/mL (140 µM) and trimipramine at 60 µg/mL (204 µM).

The dry weight of the root hairs and the number of root nodules (> 5 mm or 3–5 mm) were decreased significantly. The results demonstrated that the seedlings were more sensitive to the effect of promethazine and trimipramine than the symbiont N₂-fixing strain. The *Rhizobium*-leguminous symbiotic systems may be suitable for testing the pollution of the environment.

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Isolation and characterization of a ribosomal protein gene, *trp39* from *Trichoderma hamatum*

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The antagonistic activity of *Trichoderma* spp. is attributed to a synergistic combination of mycoparasitism, antibiosis and competition. The saprophytic competitive capability of fungal antagonists plays an important role in the colonization of the ecological niche, suppressing thus the pathogen microorganisms. Despite its importance, the mechanisms responsible for the competitive growth of *Trichoderma* spp. are not well understood.

The aim of the present study was to identify *Trichoderma hamatum* genes whose transcription could be induced by nutritional changes. By differential hybridization we created a set of clones and one of them was subjected for further characterization. This clone contained a gene (named *trp39*) encoding a 60 S cytoplasmatic ribosomal protein. Sequencing of both a genomic and a cDNA clone

predicted a 105-aa polypeptide, interrupted by two introns. The deduced TRP39 protein showed high overall identity (55–62%) to other ribosomal protein genes from *S. cerevisiae* L39 (RPL36) and mammals, including humans. High-stringency Southern analysis confirmed that *trp39* is a single copy gene. Sequence analysis of the promoter region of *trp39* revealed the presence of general promoter elements, as well as additional consensus motifs for specific functions, such as RPG-box, T-rich sequence and STRE element. The *trp39* gene showed a low level of constitutive expression when glycerol or sorbitol was used as carbon source. It was completely repressed by carbon and nitrogen starvation, by shifting up or down the temperature, as well as after the addition of 2-deoxyglucose to the cultures. In contrast, the level of *trp39* transcripts increased three to fivefold when glucose was added as a sole carbon source. The expression of *trp39* was also elevated by adding cycloheximide, 3-isobutyl-1-methylxanthine (IBMX) or a cAMP analogue, N⁶,2'-O-3',5'-cyclic adenosine monophosphate to the cultures. A similar increase of the gene expression was observed during mycoparasitic interaction, in dual culture tests.

K. FORGÁCS, B. LENKEY

**Morphological and physiological changes accompanying
the phenomenon of induced fluconazole resistance in *Candida albicans***

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We investigated a fluconazole-sensitive (MIC_{fluconazole} 5 µg/ml) clinical isolate (FS) and a fluconazole-resistant (MIC_{fluconazole} > 80 µg/ml) laboratory mutant *Candida albicans* strain (FR) developed from the sensitive one. We studied putative virulence factors including germination, adherence ability to either buccal epithelial cells or acrylate surface, the secreted aspartic proteinase (EAP), and the extracellular phospholipase (EP) activity of the two strains as well as their growth. The fluconazole-resistant strain proved to be superior in all the virulence traits tested than the original strain. The higher virulence of the fluconazole-resistant strain was also supported by mouse model. These results suggest that the development of fluconazole resistance can be accompanied by serious morphological and physiological changes: several putative virulence traits, moreover the *in vivo* virulence can increase simultaneously.

The inducible α -glucosidase is usually considered as a potential virulence factor of *C. albicans* but this opinion is not generally accepted. We measured the intracellular and intact-cellular α -glucosidase activities of the FS and FR strains grown on different

carbohydrate sources. The expression of two putative virulence factors (EAP and EP) in 20 clinical isolates of *C. albicans* in addition to the intracellular α -glucosidase activities was also investigated. Neither the physiological and enzymological studies on the α -glucosidases of the FR and FS strain nor the correlation studies between the α -glucosidase, EAP and EP activities of clinical isolates of *C. albicans* supported the view that the inducible α -glucosidase might be associated with the virulence of *C. albicans*.

Z. GAZDAG¹, T. EMRI², I. PÓCSI², G. BÖJTÍ¹, M. PESTI¹

Effect of altered glutathione and glutathione metabolism enzymes activities on chromate and cadmium sensitivity and tolerance of *Schizosaccharomyces pombe*

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A Cr(VI) sensitive mutant CS-6.51 and a tolerant mutant CT-6.66 was obtained from wild-type strain CW-6 of *Schizosaccharomyces pombe* by induced mutagenesis.

The pretreatment of strains with subinhibitory concentrations of Cr(VI) did not raise the survival rate of cells after treatment with higher concentrations of Cr(VI), in fact it was decreased in the cases of wild-type strain CW-6, sensitive CS-6.51 and tolerant CT-6.66 mutants.

The glutathione (GSH) reducing effect of Cd²⁺ is well known. The CdCl₂ pretreatment with subinhibitory concentration lowered the destructive effects of Cr(VI) at 100, 250 and 500 μ M concentrations.

The above-mentioned three strains exhibit different sensitivity to the same CdCl₂ concentration and it can be attributed to the different GSH concentrations. The tolerant mutant CT-6.66 containing the lowest amount of GSH is the most sensitive to Cd²⁺ stress.

The significantly higher GR, G6PD enzyme activities and NADPH concentration in the sensitive CS-6.51 mutant together result in an effective Cr(VI) reduction compared to the wild-type strain CW-6; and contribute to an increased generation of $\cdot$ OH.

The low GR level of the tolerant mutant CT-6.66 compared to the wild-type strain CW-6 can be explained by the low level of GSH.

The changed sensitivity that we are going to study further can be explained by the changed elements of oxido-reductive homeostasis as well as by the reactive oxygen radicals.

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Effect of altered antioxidant enzymes activities and reactive oxygen species production on stress tolerance of fission yeast

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In our experiments, Cr(VI) sensitive CS-6.51 and tolerant mutant CT-6.66 of *Schizosaccharomyces pombe* obtained by means of mutagenesis were used.

Results of the sensitive CS-6.51 and the tolerant mutants CT-6.66 are compared to that of the original parent wild-type strain CW-6. The CrO₄-isotope uptake is significantly higher in the sensitive mutant CS-6.51 than in the Cr(VI) tolerant mutant CT-6.66 and wild-type strain CW-6, where the uptake of CrO₄ isotope is approximately the same.

The high level of superoxide radical (O₂⁻) of the tolerant mutant CT-6.66 can be explained by a significantly lower mitochondrial MnSOD activity.

The unchanged activity of catalase and SOD in case of high H₂O₂ and superoxide concentration in sensitive mutant CS-6.51 can be explained by the down-regulation of these enzymes.

The response of sensitive mutants to outer oxidative stress is almost the same as that of the wild strain.

The H₂O₂ concentration and the catalase activity in tolerant mutant CT-6.66 exhibited no significant difference from those of the wild-type strain CW-6, however, the tolerant mutant showed extreme sensitivity to outer treatment with H₂O₂ and menadione.

The measurement of ·OH generation with EPR is in progress.

A. GÁCSEI, I. PFEIFFER, J. KUCSERA

Organisation of the its region in isolates of *Cryptococcus hungaricus*

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Basidiomycetes yeasts are important sources for bioactive compounds, i.e. pigments, antioxidants and lipids. The anamorphic *Cryptococcus hungaricus* described by Zsolt (1957) has reddish colonies due to carotenoid pigments of unknown constitution.

We previously reported that *C. hungaricus* strains derived from various geographic area were polymorphic evidenced by studies based on physiological, biochemical and RFLP analysis (Gácsér et al. 1999).

In the present study the ITS region of the ribosomal DNA in the strains CBS 4214, CBS 5124, CBS 6324, CBS 6569, CBS 6576, CBS 6953 were amplified by PCR. ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') were used as primers. The PCR products were subjected to restriction enzyme digestions (*EcoRI*, *HhaI*, *HaeIII*, and double digestion with *EcoRI-HhaI*). The size of the ITS regions proved to be 527 bp for CBS 4214, 559 bp for CBS 5124, 594 bp for CBS 6324, 645 bp for CBS 6569, 595 bp for CBS 6576 and 579 bp for CBS 6953. The DNA patterns of this region in CBS 6324 and CBS 6576 were identical all enzymes CBS 6569 exhibited different RFLP patterns, and significant differences in size (645 bp). The amplified ITS region of strain CBS 4214 was the smallest (527 bp), but its RFLP patterns were similar to CBS 5124, CBS 6324, CBS 6576 and CBS 6953. The nucleotide sequences of the amplified ITS regions are determined. The present study confirmed previous results, that all the strains differed from the type strains although CBS 6324 and CBS 6576 were closely related.

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The expression pattern of *cmg1*, an α -1,3-glucanase gene of the mycoparasite, *Coniothyrium minitans*

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Coniothyrium minitans is a sclerotial mycoparasite of a number of plant pathogenic fungi. Due to this capability, the fungus is used in biological plant disease control. Information on the molecular mechanisms responsible for its parasitic activity is, however rather limited. During parasitism, the fungus penetrates through the sclerotial wall that contains β -1,3-glucan as a major constituent. The production of extracellular cell wall degrading enzymes, such as chitinases and glucanases appears to

be an important factor for mycoparasitic fungi to facilitate penetration through host structures.

In the present work a PCR based strategy was applied to clone a β -1,3-glucanase encoding gene from *C. minitans*. Using degenerate oligonucleotide primers constructed on the basis of conserved regions of known fungal glucanases we isolated a full length cDNA copy of a putative glucanase gene, named *cmg1*. The nucleotide sequence of this gene and its deduced amino acid sequence was found to show high levels of similarity to fungal exo- β -1,3-glucanases. The *cmg1* gene codes for a protein of 782 amino acids. A signal peptid cleavage site is predicted between residues 24 and 25 by the SignalP program. The calculated molecular mass of the secreted protein is 82,346 Da and its estimated pI point is 4.73. The INVSc1 strain of *Saccharomyces cerevisiae*, used as heterologous expression host for *cmg1*, secreted a ~100 kDa β -1,3-glucanase enzyme (as determined by SDS-PAGE) into the culture medium. Temperature and pH optima of the recombinant enzyme purified from the yeast culture supernatant by ion exchange chromatography were 50 °C and pH 5.2, respectively. Substrate specificity and hydrolysis product analyses revealed that the enzyme is an exo-acting glucanase with high specificity to (1 $\rightarrow$ 3)- β -glycosyl linkages.

The *cmg1* gene which is present in the genome of *C. minitans* in a single copy showed a low level of constitutive expression in *in vitro* shaken cultures. It is slightly repressed by glucose and induced by carbon starvation, sorbitol, as well as ground or intact sclerotia of *Sclerotinia sclerotiorum* to a similar extent. A moderate increase in the expression of *cmg1* was observed during the parasitic interaction with *S. sclerotiorum* suggesting the role of this gene in mycoparasitism.

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Incompatibility influences the effectivity of mitochondrial transmissions

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The mitochondrial genomes of the strains *A. japonicus* belonging to the imperfect black *Aspergilli* exhibit highly variable RFLP patterns. Transmissions of mitochondria were carried out between fully incompatible mitochondrial oligomycin resistant mutant (oliR) strain as donor and sensitive recipient strains carrying mtDNAs with different RFLP patterns by using protoplast fusion technique. These transfer experiments resulted in oliR progeny with recombinant and/or unchanged donor (substituted) mtDNA in each case of intraspecific combinations, but it failed when the

closely related *A. aculeatus* was the recipient partner. Transmissions can be classified into three groups: (i) progeny with recombinant type of mitochondria only, (ii) progeny with exclusively substituted mitochondria, (iii) both types of progeny, predominantly the substituted one can be recovered. In the last case, the strains carrying substituted mitochondria display reduced fitness with appearance of decreased capacity of conidial formation. During subsequent cultivation of these oliR substituted strains a segregation process could be following resulted in morphologically stable strains with recovered fitness. These well-growing strains possess recombinant mtDNAs. With full knowledge of size differences of the donor and recombinant mtDNAs it can be concluded that intron loss itself can generate the recombinant characters. Our results might reflect the compatibility relations between strains involved in a mitochondrial transmission.

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Soil microbiological effects in long-term experiments of Keszthely and Bad Lauchstädt

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In two long-term field trials in which farmyard manure (FYM) and artificial fertilizers were compared, we analysed soil microbial biomass, respiration, organic carbon (C_o) content, total microbiological count and microbial activity (enzymatic activities for example: protease, alkaline phosphatase, β -glucosidase). The treatments included: 1. unmanured controls (O), 2. manured (FYM), 3. artificial fertilizers (NPK), 4. FYM + NPK together and different fallows (1. mechanical bare fallow, 2. bare fallow with herbicides, 3. mechanical bare fallow + herbicides and 4. green fallow).

Used methods: for enzymatic activities, for soil microbial biomass and respiration by K. Alef and P. Nannipieri (1995), for total microbiological count by R. Gahlot and N. Narula (1996), for organic carbon content by "Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlamm-Untersuchung" (1998).

The long-term experimental plots were set on in 1902 in case of Bad Lauchstädt (Germany, 11°53' East, 51°24' North, 113 m above SL) and in 1963 in case of Keszthely (Hungary, 17°15' East, 46°47' North, 112 m above SL). Both of the long-term experimental fields are used for studying the interrelation between soil, plant, microbiological flora, atmosphere and water with regard to sustainable development. The running long-term field experiments are an ideal experimental basis for this research.

Soil samples were collected in March 2000 in case of the two long-term experiments from 30 cm soil depth.

Biological and chemical measurements were made in laboratories of UFZ-Umweltforschungszentrum Leipzig-Halle GmbH, Sektion Bodenforschung, microbiological measurements in laboratory of Martin-Luther-Universität, Halle-Wittenberg, Landwirtschaftliche Fakultät Institut für Bodenkunde und Pflanzenernährung (Germany).

Our results indicate a strong correlation among the various properties. Organic carbon has positive influence on microbiological flora of various samples from the long-term experiments of both sites. Soil enzymes determined quantitatively represent various metabolic activities in combination with other properties in long-term fertilized soils and further studies will clearly show an understanding of cultivation practices, agrochemical and environmental effects in these soils.

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L. HORNOK

Research challenges for the development of genetically modified biocontrol microorganisms

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Biological control could be an environmentally feasible alternative of chemical plant protection used nowadays almost exclusively in large-scale agricultural practice. However, biological control methods should be improved in efficiency and reliability. One of the means by which the effectiveness of biological control could be increased is

the construction of genetically improved antagonistic-hyperparasitic microorganisms. In the past few years, efficient strain-breeding strategies suitable for improving plant growth promoting rhizobacteria were developed. Antagonistic and symbiotic properties were successfully combined into a single organism. Genetically engineered strains were produced to eliminate the gene drift hazards or to improve the aggressiveness of fungal hyperparasites. Although perspectives of the practical application of genetically modified biocontrol microorganisms are still uncertain, at least in Europe, a better understanding of the molecular background of the hyperparasitic way of living could help to manage and utilize the natural antagonist populations.

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**DNA sequence of a small, unidentified plasmid isolated
from a *Haemophilus somnus* strain**

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One of the plasmids present in a *Haemophilus somnus* (*H. somnus*) strain isolated from nasal discharge of a cattle with respiratory disease symptoms was purified and cloned for DNA sequencing. The copy number of the plasmid was enough to use alkaline lysis method for DNA plasmid purification, which is widely used for high-copy-number cloning vectors of *E. coli*. The plasmid was found to be 1065 base pairs long and with 39.2% G+C content. Homology search failed to find any similar DNA in the nucleic sequence data bases. The longest putative open reading frame was 261 pairs long. Based on the location of possible translation initiation codons, there is a theoretical possibility on this plasmid to code for a protein of 43 amino acids. The homology search, however, showed no significant similarity with the presently available protein data.

Presence of plasmids in *H. somnus* was detected by some authors (Fussing and Wegener, 1993; Appuhamy et al., 1998) but this is the first report on the sequencing and DNA analysis of one of the plasmids.

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Model for determination of yeast viability/mortality in the presence of chromium(VI) compounds

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The viability vs. mortality of yeasts *Saccharomyces cerevisiae* – ZIM 321 and *Schwanniomyces occidentalis* – ZIM 763 in the presence of $K_2Cr_2O_7$ was studied. After cultivating in malth broth the yeasts were exposed to the Cr(VI) in concentrations of 1, 10, 100 in 1000 $\mu\text{mol/l}$ for 24 hours at 28 °C. The survival of living cells depends on their ability to sense changes in the environment and to appropriately respond to the newly emerged conditions [1]. Stress response mechanisms aim to protect cells against the potentially detrimental effects of stress challenges and to repair any molecular damage [1]. Determination of yeast viability vs. mortality in the presence of specific effector Cr(VI) was followed in each particular step of the analytical procedure and a mathematical model-a set of equations was developed with the intention to establish a rational tool for analysing the impact of the effector. The model was built with parameters as follows: Population Condition (PC), Cell Viability in the Buffer (VB), Cell Viability in the Buffer supplemented with the Effector (VEB), Specific Cell Viability (Vs), Cell Mortality in the Buffer (MB), Cell Mortality in the Buffer supplemented with the Effector (MEB) and Specific Cell Mortality (Ms) [2]. The model was shown to be useful. It enabled to determine the difference in yeast tolerance to Cr(VI). The results showed that *Sacch. cerevisiae* – ZIM 321 is more tolerant to $K_2Cr_2O_7$ than *Schwann. occidentalis* – ZIM 763.

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I. JEVCSÁK¹, L. KÖDÖBÖCZ¹, M. KECSKÉS¹, T. SZILI-KOVÁCS², B. BIRÓ²

Microbial characteristics of pea rhizosphere affected by sewage sludge doses in some Hungarian soil types

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Municipal sludge of the Gödöllő community (Hungary) with a high Zn content was used in a long-term pot experiment to study some microbiological characteristics of the pea (*Pisum sativum* L.) rhizosphere.

Four Hungarian soil types were selected (carboneaceous chernozem Nagyhorcsók, carbonaceous sandy soil - Örbottyán, slightly acidic silty soil of Gyöngyös and acidic sandy soil of Nyírlugos). Colony forming units (CFU ml⁻¹) of some most important microbiological groups (such as *Pseudomonas*, *Rhizobium* or the total countable number) were assessed by a modified plate counting method of Angerer et al. (1998) by using Congo-red YEM, King B and Nutrient media. Biochemical characterization of the pea rhizosphere was done by chloroform fumigation procedure of biomass-C measurements (Vance et al. 1998). Metal (Zn(NO₃)₂ and ZnSO₄) tolerance of some *Pseudomonas* strains were also tested *in vitro* by using 25, 50, 100, 200, 300 µM l⁻¹ salts in three replications.

Influence of the increasing doses of communal sewage sludge on the microbiological and biochemical characteristics was highly depending on the physical and chemical properties of the soil-types investigated. There was a positive correlation between the application rates of the sludge and the total microbial counts or the microbial biomass parameters, especially in case of the nutrient poor sandy soils. There was no much relation, however, between the number of the total countable microbes and the microbial biomass measurements, due to the high dependence on the medium-components (C and N sources) in connection with the culturability.

Pseudomonas bacteria originating from the highest doses of Zn pollution showed a better *in vitro* resistance to the investigated Zn-salts, compared those strains from the unpolluted pots (in accordance with the preliminary results of Kecskés et al. 1997).

Although the total Zn concentration at the highest sewage sludge rates was still well below the international permissible standards, there was an adverse effect recorded on the selection pressure and adaptability of some beneficial microbial groups.

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Interaction between mitochondria in black *Aspergilli*

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In contrast to the nuclear genome the mtDNA does not undergo recombination process during sexual cycle owing to uniparental inheritance of extrachromosomal elements.

In case of asexual black *Aspergillus* strains bearing mitochondria with different molecular and resistance markers (such as mtDNA RFLPs and oligomycin resistance) a mixed population of extrachromosomal elements could be established under experimental conditions. Since black *Aspergilli* exhibit high degree of vegetative incompatibility their mitochondria were transferred by protoplast fusion, applying donor strains with oligomycin resistant mitochondria and sensitive recipient partner. The results of intraspecific mitochondrial transmission between *A. niger* strains, and *A. japonicus* isolates, as well as interspecific transfer between *A. niger* and *A. tubingensis* strains will be discussed. Selecting for the drug resistance and the recipient nuclear phenotype oligomycin resistant progenies were recovered. These strains basically inherited the mitochondrial DNA of the donor strain, which might remain unchanged (so-called substituted progeny) or might be modified by specific sequences of the recipient mtDNAs named recombinant mitochondria. The sequences originated from the recipient proved to be mobile introns. Substituted progeny would be either stable wild type like strain as a result of compatible co-operation between donor mitochondria and recipient nuclei, or occurred as slow growing non-conidiating strains. The latter type was probably due to the less compatible communication between two genetic systems originating from different parents. These progenies were able to undergo some segregation process during subsequent cultivation resulting in a stable wild phenotype which possessed new mtDNA type resembling to the acceptor parents. Different mobile elements characteristic for recipient parents were exclusively responsible for development of the feature of recombinant mtDNAs.

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Investigation for mould contamination of tomato purée by NIR spectroscopy

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In the industrial practice Howard mould count (1911) is used for the estimation of mould contamination of foods. It was developed originally for investigation of tomato products. It is also used nowadays for quality control purposes. Recently this constitutes the basis of the acceptance of the finished products in international trade. This technique demands experts with a lot of practice and morphological proficiency. Variation coefficient of the method is 15% or even higher.

The possibility of the determination of mould contamination of tomato puree was investigated aiming at exchanging the Howard method. The NIR technique – as a rapid, non-destructive, reagentless and accurate method – was anticipated as a suitable method for the purpose mentioned. Canned tomato puree had been allowed to become mouldy than the sample was blended with non mouldy samples in different ratio, so a series of tomato-purees containing known amounts of mouldy puree was prepared. Howard mould counts and ergosterol content – another mould contamination relating value – were used as reference values for NIR calibration.

At quantitative investigation better results were obtained using ergosterol values. The best correlation coefficient ($R=0.94$) and the smallest standard error of calibration ($SEC=0.076$ mg/g ergosterol) was achieved with triangular smoothing of the spectra. The standard error of prediction ($SEP=0.10$ mg/g ergosterol) was acceptable. At qualitative investigation Polar Qualification System (PQS) was used. Clusters between samples with low and high ergosterol levels could be separated.

K. KISS¹, K. LÁSZLÓ²

Effect of municipal sewage sludge on the phosphatase and catalase activity of Raman-type brown forest soil

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In the agricultural practice municipal sewage sludge is frequently used as a soil fertilizer. It improves the physical and chemical properties of the through the addition

of organic matter and essential nutrients, but it may cause serious ecological and hygienic problems because of its elevated concentrations of pollutants including heavy metals. The measurement of metabolic activity of microbial communities – like the measurement of enzyme activity – gives an immediate indication of the effects of perturbations on the community.

The purpose of our study was to check the effect of different concentrations of sewage sludge on the phosphatase and catalase activity of microbial communities in the soil, and to compare how can these two enzyme activities characterize the changes.

Municipal sewage sludge was obtained from the Regional Wastewater Treatment Plant, Keszthely. A subsample was digested and analysed for the total chemical content. The results: pH: 5.8, total solids: 0.10 kg/kg, N: 29400, P: 6810, K: 3470, Pb: 61, Cd 2.9, Zn: 1319, Cu: 147, Mn: 349, Cr: 30 mg/kg dry weight.

The Raman type brown forest soil was treated with different quantities of wet sewage sludge which were equal to the level of 0; 2.5; 5; 10; 20; and 40 t/ha dry sewage sludge. In small pots the samples were incubated at 25 °C for 1, 3, 8, 12 and 20 days, and the phosphatase and catalase activity were measured by photometry and titrimetry. The results were analysed mathematically.

During the whole experimental time there were significant differences at 95% confidence level in all phosphatase activity among the soils for all sludge amounts added. Expressed as percentages of control activity, the phosphatase activity ranged from 153 to 310% as for the highest concentration. Therefore the municipal sewage sludge proved to be absolutely innocuous. The results showed that the sewage sludge improved the microbial processes because of its own population and the nutrient contents.

The measure of catalase activity didn't prove to be a sensitive method for characterizing the microbial processes. There was no connection between the concentration of municipal sewage sludge and catalase activity.

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Monitoring diversity of yeast biota from vineyard to wine

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In the oenological microbiology, several publications surveyed the composition of yeast biota occurring in different vineyards and grape-processing equipments. We have a lot of information about the change of the yeast diversity at species level and the

process of their succession from must to wine. Diversity of yeasts is also big in vineyards but it is changing from field to field and from year to year. There are few results, however, about the origin of yeasts and how much they represent the yeast biota of a vineyard or cellar. One of the reasons is that the traditional identification, using morphological, biochemical and physiological tests is not suitable for characterisation at strain level. Molecular typing methods are excellent, however, for such purposes.

The aims of our research were to survey and characterize yeast biota in vineyard Etyek from the beginning of grape ripening; the study of the origin and dynamics of yeasts starting and implementing must fermentation; and to examine the interaction of the starter yeast culture and spontaneous yeast biota.

The origin of the isolates was the vineyard and pre-processing equipments in Etyek and wine factory in Budafok, using active dry *Saccharomyces cerevisiae* starter cultures. We collected samples before and after vintage, and followed the spontaneous and inoculated fermentations. Our isolates were identified at species level by traditional identification methods. The similarity of the strains and typing at species level were examined by molecular genotyping methods, such as RAPD-PCR, pulsed field gel electrophoresis and RFLP of rDNA sequences.

Several yeasts were found in the cleaned equipments before vintage, which probably survived there. The basidiomycetes *Cryptococcus laurentii* and *Sporidiobolus salmonicolor* were dominant.

The number of yeast cells increased by about two-three order of magnitude during vintage but the wide biodiversity still remained. *Sp. salmonicolor* still occurred among them, but the ascomycetes *Debariomyces*, *Hanseniaspora* and *Saccharomyces* genera became dominant.

In the grape juice *Hanseniaspora uvarum* was the dominating species and we found several *Saccharomyces ludwigii* and *Candida apicola* isolates, too. At the beginning of fermentation, *Torulaspora* occurred in the highest ratio, beside the dominant *Saccharomyces sensu stricto* species. In the middle and last fermentation phase, we found *Saccharomyces sensu stricto* species only.

With the application of molecular typing methods, we determined the similarity of strains belonging to a given species, and detected yeasts strains, which survived in the processing equipments. These methods proved very useful in monitoring the succession of yeasts during spontaneous and inoculated fermentations.

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Comparison of the antibiotic-sensitivity of lupine rhizobia originating from different agrotechnical systems

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Various agrotechnical conditions (such as organic and conventional) may influence the functioning and stress-tolerance ability of the different soil-plant systems. Among the main environmental factors the antibiotic sensitivity may reflect the interactions of the microbial populations.

30 *Bradyrhizobium* sp. strains, originating from the root-nodules of sweet lupine (*Lupinus albus* L.) were investigated for their antibiotic sensitivity in *in vitro* conditions. 19 strains were isolated from the traditional crop-rotation experiment of Westsik, started in 1929 on the slightly acidic sandy soil of Nyírség (Lazányi, 1994). Other strains were originating from the same hosts, grown in an intensive agricultural system (with the originally high amounts of agrochemicals and fertilizers). Yeast-mannitol-agar (YMA) plates were used to study the sensitivity of seeded lupine rhizobia (100 µl of 10⁶ CFU ml⁻¹) against 10 different antibiotics (RESISTEST discs) in 3 replicates. After 24 hours of incubation at 28 °C, the inhibition zones were registered (Vincent 1970). Results were subjected to two ways of ANOVA investigations.

A much higher availability and dimension of antibiotic sensitivity was found with the 19 *Bradyrhizobium* strains of the Westsik organic crop-rotation systems. This phenomenon was highly influenced by the different organic treatments of the XV plots, which has resulted a great variation of the general soil conditions, as a consequence. Biomass production, symbiotic characteristics and nitrogen-fixing effectiveness produced the same diversity of data in an earlier study (Ködöböcz et al. 1999).

In the intensive agrotechnical systems, therefore, the isolated lupine rhizobia were found to be identical for the general antibiotic sensitivity, which reflects a reduced diversity of the population and the selection of antibiotic-resistant lines. Westsik organic treatments, however have preserved the natural genetical balance of the lupine rhizobia. Interrelations between the antibiotic sensitivity and the nodulation and nitrogen-fixation ability is further discussed.

Relevance of the organic systems to preserve the natural biodiversity of beneficial microbes (nitrogen-fixing rhizobia) is highlighted from this study.

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**Influence of heavy metals on mycelial growth and *in vitro* enzyme activities
of mycoparasitic *Trichoderma* strains**

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The effect of ten heavy metals (aluminium, copper, nickel, cobalt, cadmium, zinc, manganese, lead, mercury and iron) on the linear mycelial growth, and on the *in vitro* activities of extracellular enzymes involved in antagonism against plant pathogenic fungi (β -glucosidase, cellobiohydrolase, endocellulase, β -xylosidase, endoxylanase, β -1,4-N-acetylglucosaminidase, trypsin-like protease, chymotrypsin-like protease, and β -1,3-glucanase) was studied in case of six *Trichoderma* strains belonging to species groups *T. harzianum*, *T. viride* and *T. aureoviride*. Each particular heavy metal inhibited mycelial growth in all six strains to a similar extent, significant variation of IC₅₀ concentrations between the strains was found only in case of iron. In a concentration of 1 mmol, only mercury showed high inhibitory effects on the *in vitro* activities of the investigated enzymes, the inhibition of enzyme activities by the other heavy metals was not so expressed. However, the slight inhibition of trypsin- and chymotrypsin-like activities by aluminium, copper, and lead should be mentioned. Extracellular enzymes seem to remain active even at heavy metal concentrations, where mycelial growth is already strongly inhibited. This suggests the possibility of breeding for heavy metal resistant *Trichoderma* strains for biocontrol purposes in soils with heavy metal contamination or for combined application with heavy-metal containing pesticides.

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Effect of nisin on the microbial stability of sausages

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The antimicrobial effect of nisin produced by *Lactococcus lactis* is well known. Nisin is the only bacteriocin that has been accepted as a preservative in some foods. Since consumers are oriented to natural food products, preserved with less or no chemicals, the use of natural preservatives is in the centre of interest.

The antimicrobial effect of nisin in different meat products was studied. Nisin was added to meat product (Bologna-type sausage emulsion) and experiments were performed to determine the effect of nisin for the microbial stability and sensory characteristics.

According to the results we concluded that nisin in 100 and 200 IU g⁻¹ concentration extended the shelf life of these products, without nisin the total viable cell count and enterococci were high after 30 days of storage. By use of these concentrations off-flavour of the samples were noted. The use of nisin in lower concentration (50 IU g⁻¹) also resulted a low microbial viable cell count and without any off-flavour.

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Colony variation of the human pathogenic yeast *Cryptococcus hungaricus* on Phloxin B medium

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Cryptococcus neoformans is an encapsulated opportunistic human pathogenic yeast, which can cause meningitic, pulmonary or systemic infections. It can occur in immunocompetent patients but it is more frequent and cause more severe symptoms in patients with acquired immunodeficiency syndrome (AIDS) or other conditions associated with immunosuppression. Cultivation of *C. neoformans* isolates on complete medium containing 10 µM Phloxin B resulted in different coloured colonies (pink, red and sectors of both pink and red). Colony-colour variants were detected in two laboratory strains (IFM 5844, 5845) but not in 20 other strains derived from AIDS patients. Segregants were isolated from the red (αR) and pink (αP) sectors of one

colony from IFM 5844. Beside these original isolates an additional slow growing colony was isolated from a subculture of α R (α Rs). These three isolates were analysed for physiological and molecular peculiarities. The segregants differed in the size of capsule and also in growth rate and temperature tolerance. Karyotyping by CHEF demonstrated that there were no chromosomal polymorphisms among them. However minor genetic polymorphisms were evident in the DNA fingerprints obtained by RAPD PCR among the three segregants. The process by which red (hypocapsulated) and pink (highly capsulated) colour variants arose could be attributed to phenotypic switching as it was shown by reversion frequency analysis. The plausible explanation for temperature sensitive variant was random spontaneous mutation, as reversion to growth at 37 °C was not observed among cells of the temperature sensitive segregant α Rs. The reason for colour changes was also investigated and attributed to the differential ability of the cells to accumulate the xanthene dye Phloxin B either into their capsules or into their cells. The cultivation method described here is potentially applicable for detection of strain heterogeneity and varying degree of encapsulation in both clinical and basic microbiology laboratories.

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Cloning and heterologous expression of cell wall degrading enzyme encoding genes of *Thermobifida fusca*

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Twelve thermophilic actinomycete isolates were screened for cell wall degradation activity. Among them *Thermobifida fusca* strain TM51 proved to be the most active cellulase and xylanase producer. *Streptomyces lividans* strain TK24 was used for the expression of the *T. fusca* hydrolase genes. This closely related host possesses the appropriate protein folding, glycosylation and secretion system for the heterologous enzymes. A genomic library was constructed by using the plasmid pIJ699. Genomic DNA fragments with an average size of 15 kbp were ligated into pIJ699. The transformation frequency approximated 90% and altogether 1700 thiostreptone positive colonies were isolated. This amounts to a 93% representation of the complete gene bank.

S. lividans strains harbouring the recombinant plasmids were grown on liquid LB medium, which prevents the induction of the host's own polysaccharide degrading enzymes. After 48 h micro-fermentation period the individual transformants were

screened for enzyme activity by means of a fluorogenic substrate-mix containing methylumbelliferyl- β -D-glucoside, -mannoside, -cellobioside and -xyloside. Endoglucanase and endoxylanase activities were measured by the Congo-red staining method. Crude enzyme preparations were obtained from the positive transformants and subjected to SDS-PAGE zymogram analysis. Screening of the expression library yielded a number of *T. fusca* hydrolases, including five different endoglucanases (their molecular weights ranged between 45 and 110 kDa), two endoxylanases (48, 53 kDa), one β -mannosidase (75 kDa), a cellobiohydrolase (62 kDa) and a β -xylosidase (130 kDa). This latter proved to be a dimeric enzyme, as after heat shock treatment it was dissociated to two equal, 66 kDa monomers resulting in the total loss of enzyme activity.

The extreme heat stability and detergent tolerance of the two endoglucanases are worthy of mentioning. These enzymes retained their activity after 5 minutes boiling in loading buffer containing 1% SDS and 100 mM mercaptoethanol, indicating their potential utility for industrial application.

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***Thermobifida cellulolytica* sp. nov., a new lignocellulose decomposing
member of the genus *Thermobifida***

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Five actinomycete strains, K21, K52, TB100, TB108 and TB110, were isolated from the overheated region of manure compost. The isolates were assigned to the genus *Thermobifida* on the basis of morphological, physiological and biochemical characteristics. All investigated strains produced single, ovoid (11.4 μ m), heat sensitive (wet heat, 80 °C, 15 min) spores on dichotomically branched aerial hyphae, as revealed by scanning electron microscopy. The temperature range of growth was 35–45 °C for the thermotolerant strains, K21 and K52, whereas this value ranged between 42–65 °C for the thermophilic isolates, TB 100, TB 108 and TB 110. All the strains were capable of degrading starch, xylan, as well as soluble and crystalline cellulose; they showed lignocellulose solubilization on straw powder. They tolerated erythromycin at concentrations of 5–50 μ g ml⁻¹, whereas *Thermobifida fusca* strains were sensitive to this antibiotic. The partial 16S rDNA sequence similarity among these newly isolated

strains reached 99.8–100%, whereas the almost complete 16S rDNA sequence of TB100, the representative strain of this collection, showed only 97.4 and 97.8 similarity to the corresponding rDNA sequences of the type strains of *T. fusca*, and *T. alba*, respectively. The 70–71 mol% G + C content of the DNA of these isolates significantly differed from the 66.5 mol% G + C value determined for the type strain of *T. fusca*. The chemotaxonomic features of these isolates were similar to that of the well documented species of the genus *Thermobifida*. Cell wall analysis revealed the presence of meso-diaminopimelic acid, but no other characteristic amino acids or sugars in the mureine (cell wall type III). According to polar lipid analysis all strains showed PLII type phospholipid composition; phosphatidylethanolamine was detected together with some unidentified phospholipids. The cellular fatty acid and isoprenoid quinon composition of the new isolates characteristically differed from that of the other two *Thermobifida* species. Based on both phenotypic and genotypic data we suggest that these strains should be assigned to a new species within the genus *Thermobifida* under the name *Thermobifida cellulolytica* sp. nov. The type strain is TB100.

L. MAJOROS, C. MISZTI, G. KARDOS, I. ANDIRKÓ, B. SZABÓ

Fungal sepsis in newborns and childhood

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We have studied *Candida albicans* sepsis of two stillborns (below 1000 gram) and of a 3-years-old child. Invasion of *C. albicans* into cerebrospinal fluid (CSF) was observed in case of two newborns. The culture from blood and CSF of the boy patient gave positive result in 3 cases. The samples from the respiratory tube proved to be positive for the presence of *C. albicans* in 3 cases and in 1 case this fungus was isolated from the purulent arthritis as well.

The ineffective fluconazole therapy was replaced by liposomal amphotericin-B treatment for 3 weeks. This appropriate therapy resulted in complete healing of arthritis as well.

The granulomatoid meningitis of the newborn girl was fatal. In addition to positive CSF culture before the death the hyphal form of *C. albicans* was also demonstrated from the brain after autopsy.

The pulse-field electrophoresis revealed the same karyotype pattern of 7 *C. albicans* isolates of the newborn boy indicating the generalization of the infection. Similarly, the CSF and blood *C. albicans* isolates of the newborn girl were also identical.

The *C. albicans* sepsis of a 3-years-old girl after operation a Fallot-tetralogy was of a better outcome. Three days after the operation *C. albicans* was cultured from the pleural fluid. This finding was followed by 12 additional identical isolations of *C. albicans* (hemocultures, respiratory tube, wound).

The adequate amphotericin-B therapy completely eradicated the fungal infection.

In vitro and *in vivo* studies are going on in our laboratory to investigate the potential neurovirulent nature of our *C. albicans* isolates.

L. MAJOROS, C. MISZTI, G. KARDOS, I. ANDIRKÓ, B. SZABÓ

Repeated positive Candida cultures from male urine samples

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During a 29 month period the Laboratory for Bacteriological Diagnostics of University of Debrecen tested about 70 thousand specimens. Five hundred seventy-one samples proved to be positive for the presence of yeasts from 11 062 urine specimens. The etiological connection between candidurias and kidney lesions requires support by repeated cultivation of *Candidas* mainly in females but in males as well. Because of the anatomical differences the possibility of contamination is significantly lower in males, thus the subject of the present study was the male candiduria. In 33 cases two or more urine samples were positive for the presence of *Candidas* during repeated sampling.

We have obtained the repeated urine samples from four different groups of patients. In the first group of 6 renal transplant patients *C. albicans* was cultured from blood or ascites fluid as well. In the second group of 6 patients from Department of Urology we isolated from one patient *C. glabrata* 12 times. We have isolated repeatedly *Candidas* from urine of young children (7 boys). The hospitalized, burned patients represented the fourth group of our patients who received antibiotic treatment for a long time. Repeated positive cultures were also registered in this group.

In our study groups the invasive instrumental procedures, the catheters, the anatomical situations, the immunosuppressive therapies and/or conditions make plausible the etiological relationship between candiduria and the disease.

The pulse-field electrophoresis method was useful for determination of karyotype profile of the *Candida* strains. In case of identical karyotype of the repeatedly isolated *Candida* strains the above discussed etiological connection seems to be much more plausible.

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**Species diversity of bacteria participating in the biodegradation
of reed rhizomes**

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Traditional cultivating procedure was used to study the bacterial community structure developing on the surface of the degrading reed rhizomes. The rhizomes were exposed to biodegradation in net bags on the water surface of their natural environment, a sodic steppe lake. For the isolation and cultivation of bacterial strains cellulose containing oligotrophic, complex and alkaline media were applied.

The bacterial strains based on their phenotypic characteristics were grouped into different phenotypes from which representatives were chosen to ARDRA. The members of each ARDRA groups were subjected to partial sequencing of 16S rDNA.

The strains which were isolated from cellulose containing synthetic medium and possessed cellulolytic activity were identified as: *Paenibacillus illioniensis* (97.2%), *Bacillus benzoovorans* (96.4%), *Cellulomonas favigena* (96.2%), *Methylobacterium organophilum* (94.9%), *Rahnella aquatilis* (99.8%), *Rhodopseudomonas acidophila* (93%), *Pseudomonas citronellolis* (96.0%) and *Agrobacterium tumefaciens* (96.7%).

The results of the partial sequencing of 7 bacterial strains originated from the cellulose containing alkaline media were as follows: *Exiguobacterium aurantiacum* (98.6%), *Dietzia* sp. (99.7%), *Bacillus alcalophilus* (97%), *Nesterenkonia halobila* (95.4%), *Halomonas venusta* (96.8%), *Pseudomonas oleovorans* (93.0%) and *Stappia stellulata* (93.7%).

I. MIKLÓS, M. SIPICZKI

**Suppression of a cytokinesis defect in a *Schizosaccharomyces pombe*
mutant strain**

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Study of cell cycle is a favourite topic of cell biology as different defects of cell division can cause for example cancer.

We are interested in cytokinesis (separation of mother and daughter cells), which is the last step of cell division. The fission yeast *S. pombe* was used for the experiments, which is a genetically tractable model system. Since it is a unicellular

microorganism, the failure of cell separation causes the formation of threads, which contain several cells.

Lots of strains defective in cytokinesis were isolated. One of them was referred to as *spl1-1* (sep-like Sipiczki et al. J Cell Sci 1993).

Investigation of the *spl1-1* strain shows that mutation in the *spl1*⁺ gene confers a pleiotropic phenotype to the cells:

- longer, slow growing cells even at 30 °C,
- mycelial bent cells, which cannot separate from each other at the restrictive temperature (35 °C),
- temperature sensitivity, as the cells stop growing with diffuse nuclei and 2C DNA content at restrictive temperature,
- the cells are sensitive to CaCl₂ and caffeine.

To determine the precise role of the *spl1*⁺ gene, we started to clone the gene. Up to the present we have managed to find just a suppressor, which is a proline t-RNA gene.

Further experiments to find the genomic *spl1* gene are under way. The mutant strain was transformed with a wild-type genomic library. One positive clone has been found and it is being sequenced.

CS. MOHÁCSI-FARKAS¹, L. MÉSZÁROS², J. FARKAS², G. KISKÓ¹

Non-thermal pasteurization of tomato juice by high hydrostatic pressure treatment

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In the frame of an INCO/COPERNICUS EU ("Plantchem") project antimicrobial activity of essential oils of medicinal and aromatic plants and their potential application as biopreservatives were tested. In addition, the novel non-thermal pasteurization treatment, the high hydrostatic pressure and their combinations were investigated.

Freshly prepared tomato juices were treated in several experimental series with addition of essential oils and/or by high hydrostatic pressure. High hydrostatic pressure ("HP") treatments were performed by a Stansted (U.K.) "FoodLab-9000" high hydrostatic pressure equipment. The samples were stored at 15 °C, and periodical microbiological testings were performed during storage.

Both pressure treatment of 400 MPa for 20 min and addition of 0.5% oregano or thyme oil were able to result in a microbiologically stabile product for at least a few weeks.

Significant retardation of lactic acid spoilage was obtained even by 0.05% level of oregano oils whereas a full microbiological stability of the tomato juice was achieved by the higher pressure for a shorter time (400 MPa, 5 or 10 min) HP-treatment.

Addition of 0.05% essential oils (thyme and dill seed) had only little effect on the microbiological growth when applied alone and the efficiency of the relatively mild, 200 MPa, 10 min pressure treatment could not be increased by this low level of essential oils, resulting only in a temporary delay of spoilage.

The microbiological shelf-life of the tomato juice was extended and the efficiency of the 200 MPa, 10 min pressure treatment was increased considerably by the addition of 0.1% thyme oil resulting in a microbiologically stable product for at least 3 weeks of storage at ambient temperature investigated. Thus, combining the two non-thermal preservative methods makes it possible to reduce the concentration requirement of essential oils and/or the requirement (pressure or duration) of the high hydrostatic pressure treatment.

Z. NAÁR¹, F. ROMÁN², M. KECSKÉS²

**Long-term effect of heavy metals on the species composition
of *Trichoderma* population in a chernozem soil**

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Indigenous species of the *Trichoderma* population was studied in a long-term field experiment, where calcareous chernozem soil was artificially polluted with heavy metals 7 years prior the assessment. Three doses (30, 90, 270 mg kg⁻¹ dry soil) of the Cd, Ni and Zn salts (as sulphates) were studied on the species composition of *Trichoderma* fungi in parallel with the estimation of arbuscular mycorrhizal colonizations (Vörös et al., 1998). A *Trichoderma* selective medium (Askew and Laing, 1993) was used for the artificial cultivation of small soil particles for 1 week at 28 °C. Abundance of the various species was estimated by microscopical identification of isolates according to Rifai (1969), Bissett (1991a-c, 1992). Correlation-regression analysis was used to estimate the statistical interrelationships between the available heavy metal content and the diversity and abundance of *Trichoderma* population.

Six *Trichoderma* species (*T. atroviride*, *T. harzianum*, *T. pubescens*, *T. tomentosum*, *T. virens* and *T. viride*) were isolated from the untreated, control soil at various frequencies. Heavy metal pollution in general has resulted in significant loss both in the diversity and in the abundance of *Trichoderma* population. All heavy metal treatments have decreased the Shannon's diversity index. In case of Cd, however, there was a slightly increased diversity found at the Cd-90 kg ha⁻¹ rate, preceding the total loss of the *Trichoderma* population at the highest dose. There was such a tendency published by Bíró et al. (1999) in case of As and Se toxic metals in the same long-term experiment. The highest number of *Trichoderma* propagules was found at the 270 mgkg⁻¹ doses of Ni and Zn heavy metals.

A reduced correlation is concluded for the species composition of the *Trichoderma* fungi at the lower doses of heavy metal salts. Simultaneously with the toxicity, however the role of heavy metal salts are increasing. Correlation regression analysis proved to be a suitable tool for estimating the interrelations between the *Trichoderma* species.

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Pulsed field electrophoresis of *Mortierella* (*Micromucor*) chromosomal DNA

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Many members of *Mortierella* genus are potent producers of long chain polyunsaturated fatty acids (e.g. arachidonic and dihomo- γ -linolenic acids). These compounds are important both nutritionally and pharmacologically. In spite of their biotechnological importance, the organization of the nuclear genome has not been described so far.

Electrophoretic karyotype analysis using different approaches of pulsed field gel electrophoresis has led to a significant progress in fungal genetics. In the present study,

contour-clamped homogeneous field pulsed-field electrophoresis (CHEF) was applied to obtain information on the organization and intragenetic variability of the nuclear genome in three *Mortierella* (*Micromucor*) strains from different species (*M. isabellina* and *M. ramanniana*). None of the strains gave the same electrophoretic pattern; 12 (for *M. isabellina*) and 11 or 14 (for *M. ramanniana*) chromosomal mobility groups were resolved. The relative intensities of the separated bands suggest the presence of several comigrating chromosomal DNAs, resulting in comparable genome sizes (about 24 Mb) for *Mortierella*, which are larger than those reported for other yeast genomes, but smaller than those observed for the majority of the filamentous fungi. Gene assignment experiments with cloned heterologous gene probes were also carried out.

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**Carbon catabolite repression of β -galactosidase biosynthesis
by *Penicillium chrysogenum***

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Industrial production of penicillin by *Penicillium chrysogenum* is carried out with lactose as the carbon source by feeding subrepressing doses of glucose since the production of penicillin appears to be favoured by suboptimal growth conditions. Carbon source regulation seems to act at several points of the penicillin biosynthesis. Carbon catabolite regulation of penicillin biosynthesis is exerted by glucose and other easily utilizable carbon sources but not by lactose. Investigating the carbon catabolite repression in *Penicillium chrysogenum*, a similar phenomenon was observed in case of β -galactosidase biosynthesis. Using lactose as the only carbon source in shaken cultures of *Penicillium chrysogenum* the β -galactosidase activity is the highest. Addition of glucose to the culture reduced the biosynthesis of the enzyme. Other repressing carbon sources such as sucrose, galactose and glycerol also have a negative effect on β -galactosidase production. Lactose induced the biosynthesis of β -galactosidase, glucose and other easily utilizable carbon sources repressed it. Glucose grown cultures had no β -galactosidase activity but after consuming of the glucose enzyme activity increased. This phenomenon is due to the derepression of enzyme synthesis. Adding caffeine to lactose+glucose grown cultures had no effect on enzyme synthesis but significantly decreased the growth of the cultures. This can be explained

if the increased intracellular cAMP concentration had no effect on catabolite repression, but reduced glucose utilization.

QUANG D. NGUYEN, J. M. REZESSY-SZABÓ, Á. HOSCHKE

Improvement of amylolytic production by changing the components of fermentation medium

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One of most important point of view in fermentation industry is to apply the suitable medium components. In this way, not only the amount of the final product can be increased, but it is also possible to reduce the expenses of fermentation medium. The effects of carbon- and nitrogen-sources and the initial pH of medium on the amylolytic activities of *Thermomyces lanuginosus* was investigated in this study.

According to previous reports the amylolytic enzymes are stable at pH ranging from 4.0 to 8.0, the optimum pH is between 4.6 and 6.5. Based on preliminary experimental results we confirmed that the pH of the ferment broth reached very high values (pH=8.0–9.5) at the end of the fermentation, which may cause the inactivation of the enzymes. The effects of initial pH of media were examined by preparing them with 100 mM citrate buffer of different pH. The analysis of the changes of pH values during fermentation showed that the pH in the reference trial increases to a great extent. The application of a buffer system could restrict the increase of pH. The results revealed that the pH has strong influence on amylolytic activities. When the initial pH of the media was adjusted to 4.9 with citrate buffer, the glucoamylase activity was 3.5 times and the α -amylase activity 1.7 times higher than that of the reference trial. In case of buffered conditions the profile of the dry mass and amylolytic activities showed inverse function with the initial pH of the medium. In this way it was possible to repress the mycelium growth and favour the enzyme production. If the pH of the medium was lower than 4.0 both amylolytic activities decreased drastically. It should be noticed that if the initial pH ≤ 3 no growth was observed.

The effects of various carbon-sources (starch, glucose, maltose, maltodextrin, amylopectin, amylose, dextran and dextrin) on the production of amylolytic enzymes were investigated. Starch is generally accepted as nutritional component for induction of amylolytic enzymes. In our study this was applied as reference. Growing fungus on glucose, maltose, maltodextrin, amylopectin, dextran and dextrin the amylolytic activities were higher than on starch. In case of α -amylase the maltodextrin was found to be the best carbon source. Applying maltodextrin as a sole carbon source the α -

amylase activity was approximately 25% higher than that of the control one. In case of glucoamylase the dextrin proved to be the best, giving twice higher activity than starch.

In the investigation of the effects of various nitrogen-sources (L-asparagine, yeast extract, $\text{CH}_3\text{COONH}_4$, NH_4NO_3 , NaNO_3 , $(\text{NH}_4)_2\text{SO}_4$ and $\text{NH}_4\text{H}_2\text{PO}_4$) on amylolytic enzyme production, L-asparagine was found to be the most promising one. In case of α -amylase activity yeast extract seemed to be suitable as well, but in case of glucoamylase the enzyme productivity was only half of that, which was produced on L-asparagine. When inorganic nitrogen-sources were used, the fungus grew excellently, but very poor enzyme activities were achieved.

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Taxonomic position of *Gilbertella persicaria* based on glyceraldehyde-3-phosphate dehydrogenase gene and nuclear ribosomal-DNA sequence data

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Gilbertella persicaria (a member of the Mucorales) is known to be as a storage-rot microorganism primarily of peaches. This species was originally described as *Choanephora persicaria* and was included in the Choanephoraceae on the basis of the morphology of the sporangia and sporangiospores. Later, the genus *Gilbertella* was introduced and placed into the Mucoraceae, because this fungus forms *Mucor*-type zygospores. Finally, this genus (and *G. persicaria*) was removed to a newly-created monogeneric family (Gilbertellaceae). The first objective of this study was to reveal the phylogenetic relationships between fungi belonging to Gilbertellaceae, Choanephoraceae and Mucoraceae. Two strains of *G. persicaria*, 7 strains belonging to the Mucoraceae (*Actinomucor elegans*, *Mucor piriformis*, *M. rouxii*, *M. circinelloides*, *Rhizomucor miehei*, *R. pusillus* and *R. tauricus*) and 4 strains of the Choanephoraceae (*Blakeslea trispora* (+), *B. trispora* (-), *Choanephora conjuncta* and *Poitrasia circinans*) were involved in these experiments. The complete ITS (internal transcribed spacer) region coding the ITS1, the ITS2 and the 5.8S rDNA and a fragment of the glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) consisting of 240 base pairs were amplified by polymerase chain reaction. Sequences of the amplified DNA

fragments were determined and analysed. The congruency of the sequence data obtained by the analysis of the ITS region and the *gpd* gene was also evaluated. The results support the "intermediate" position of *G. persicaria* between Choanephoraceae and Mucoraceae.

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The influence of chromium compounds on yeast viability

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The intrinsic, biochemical, structural and genetic properties of the yeast cell along with environmental conditions are crucial for its survival when exposed to toxic metals (1). The ability of yeasts to tolerate higher concentrations of chromium, which plays an important role in their metabolism (2), depends on the concentration and form of the chromium compound. The effect of chromium(VI) compounds (K_2CrO_4 , $K_2Cr_2O_7$, $Na_2Cr_2O_7 \cdot 2H_2O$) in concentrations of Cr(VI) 0.001, 0.01, 0.1, 1 mM and chromium(III) compounds ($CrCl_3 \cdot 6H_2O$, $Cr(CH_3COO)_3$, $KCr(C_2O_4)_2 \cdot 3H_2O$) in concentrations of Cr(III) 0.1, 1, 10, 100, 200 mM on the viability of yeast *Saccharomyces cerevisiae* ZIM 753 was studied. After cultivation in malt broth the biomass was harvested in the early stationary phase, washed and resuspended in MES buffer (pH=4.0) and adjusted to dry mass concentration of 0.05 mg/ml. The effect of chromium compounds on the yeasts was lasting 24 hours in the MES buffer at 28 °C. The yeast viability was determined as CFU on agar plates. The results showed that different chromium compounds exhibit different toxicity to the yeast *Saccharomyces cerevisiae*. Yeasts tolerated Cr(III) compounds more efficient than Cr(VI) compounds (3), furthermore, there were also differences in toxicity among all compounds. The highest toxicity was observed in case of $CrCl_3 \cdot 6H_2O$ among Cr(III) and in case of $K_2Cr_2O_7$ among Cr(VI) compounds. However, the highest tolerance was found in the presence of K_2CrO_4 for Cr(VI) and $KCr(C_2O_4)_2 \cdot 3H_2O$ for Cr(III).

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A. PENGOV

Bovine mycotic mastitis

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Objectives: The objective of this study was to evaluate the prevalence of yeast udder infections among dairy cows.

Material and methods: The following study includes a total amount of 93 selected isolates obtained from culture of milk samples from dairy cows. During a six-month period milk samples of 1652 cows functional quarters were taken.

The above-mentioned 93 isolates were differentiated with the API 20 C and API C AUX (Bio Merieux) systems.

Results: Besides bacterial udder pathogens 76 yeast strains and 17 prototheca were isolated from infected bovine mammary glands. The most prevalent species were:

Candida krusei, *C. glabrata* and *C. albicans*.

No relation was found between the different species in regard to the somatic cell count and the severity of clinical symptoms.

I. PFEIFFER, J. KUCSERA

Organization of the mitochondrial genome in *Phaffia rhodozyma* CBS 5905

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While the structure of the mitochondrial genome seems to be circular in the majority of cases, some organisms have linear mitochondrial DNA. Independently of the structure one intact molecule specifies several proteins of the respiratory complexes, two ribosomal RNA and tRNAs characteristic of the species.

Phaffia rhodozyma is a red-pigmented heterobasidiomycetous yeast species [1]. The existence of its linear DNA plasmids was proved in 1987 [2]. Our study demonstrated that these plasmids or some of them represent the mitochondrial genome of this species. The type strain of *Phaffia rhodozyma* (CBS 5905) does not contain DNA plasmids. The molecular weight of its mtDNA was calculated by restriction enzyme analysis and proved to be approximately 13 kb. Its structure was analysed by electron microscopy. Mitochondrial genes from different fungi do not hybridise to it,

for this reason homologous gene probes were prepared by PCR techniques. The partial sequence of the *coxI* and *ATPase9* was determined.

This strain gives small, "petite" colonies with low frequencies among the normally growing ones. These colonies fermented glucose in the presence of oxygen, were not able to grow on nonfermentable carbon sources and did not respond to inhibitors of respiration. Besides spontaneous occurrence, this mutation proved to be almost 100% inducible by low levels of ethidium bromide treatment, suggesting that it is mitochondrially inherited.

According to our results, *Phaffia rhodozyma* is the only known petite-positive basidiomycetous yeast and has the smallest mitochondrial genome among fungi.

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I. PÓCSI, T. EMRI, L. SÁMI, T. PUSZTAHELYI

The glutathione metabolism of the filamentous fungus *Penicillium chrysogenum*

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Glutathione (γ -L-glutamyl-L-cysteinyl-glycine; GSH) is structurally analogous to δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine (ACV), which is an important intermediate of the β -lactam biosynthesis, and, as a result, GSH suppresses the penicillin production by inhibiting ACV synthase and isopenicillin N synthetase (IPS) activities in *P. chrysogenum*. Detailed physiological studies on *P. chrysogenum* revealed that

1. The *P. chrysogenum* tolerated the oxidative stress caused by high concentrations of peroxides (up to 700 mM H_2O_2 or 2.0 mM tert-BOOH) exceptionally well. This resistance was explained by the high intracellular glutathione peroxidase and catalase activities.

2. An idealistic culture media for β -lactam production by *P. chrysogenum* would contain lactose, glutamate and cysteine as carbon, nitrogen and sulphur sources,

respectively. Nevertheless, all of these additives would increase the intracellular GSH concentrations too and, hence, should decrease the penicillin yields.

3. Nevertheless, under β -lactam producing conditions the intracellular GSH pool can be decreased considerably via the GSH-dependent detoxification of the highly toxic, protonophore penicillin side chain precursors phenoxyacetic (POA) and phenylacetic (PA) acids. So, in an indirect way, the side chain precursors themselves could release the penicillin biosynthetic machinery from the inhibitory effect of GSH.

In addition, possible connections between the acidity, toxicity, GSH-dependent detoxification and β -lactam production of POA, PA and their hydroxylated derivatives were also thoroughly investigated. The toxicity of the side chain precursors correlated with GSH-dependent detoxification but did not correlate well with acidity and β -lactam production. Thus, penicillin production does not seem to be an alternative of the GSH-dependent detoxification. Moreover, reactive, epoxide intermediates of the hydroxylation reactions are thought to contribute profoundly to the toxicity of the side chain precursors in addition to their protonophoric character. These intermediates are the most probable targets for the GSH-dependent detoxification.

The senescence in ageing *P. chrysogenum* cultures can be characterized with fragmentation, the release of hydrolytic enzymes, autolysis and, at last, the onset of a new equilibrium between cell dying and cryptic growth. From these processes, fragmentation and cryptic growth can be hindered using the pseudotrisaccharide allosamidin, one of the most potent and specific inhibitors of chitinases. On the other hand, neither the specific GSH concentrations nor the GSH/GSSG redox balances of the *P. chrysogenum* cells were disturbed by either ageing or the inclusion of allosamidin into the culture media. Hence, any causal connection between the observed morphological changes and the redox status of the cells seems to be unlikely.

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Yeast biomass enriched with chromium

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Chromium, in trace amounts, is an essential component of human nutrition and it has been determined to be required for normal carbohydrate and fat metabolism. Beside foods naturally rich in chromium, brewer's yeast is the good natural source, but chromium concentration is quite low ($0.2\text{--}5\ \mu\text{g g}^{-1}$ dry biomass). The aim of our study was to prepare yeast biomass enriched with chromium. It is known that microorganisms are able to accumulate toxic metal ions such as hexavalent chromium.

The screening method according to tolerance to Cr(VI) among nine yeast strains was performed. The most tolerant yeast was *Candida intermedia* ZIM 156, which was further cultivated with chromium chloride hexahidrate compound. It was established that this yeast could accumulate Cr(III), which is desirable from the nutritional aspect. Investigation of some ecological parameters with intention to reach high accumulation of chromium in the yeast biomass was done. Beside addition of chromium at different phases of yeast growth two physicochemical parameters, like concentration and temperature on chromium accumulation, were investigated. In case of concentration (3, 5, 10 mM Cr³⁺) the results showed that yeast growth at higher chromium concentration was more inhibited but accumulation of total chromium was higher, which corresponded to 670, 960 and 2400 µg g⁻¹ dry biomass, respectively. Addition of chromium (5 mM Cr³⁺) in the middle of the exponential phase did not show the inhibitory effect on yeast growth, but accumulation was lower (610 µg g⁻¹ dry biomass). Increase of temperature in the deceleration phase had an additional effect on chromium accumulation, which was 920 µg g⁻¹ dry biomass.

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Accumulation of chromium(III) in yeast *Candida intermedia*

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Chromium is an essential microelement for mammals and probably for microorganisms and it is most notably associated with glucose metabolism. Inorganic chromium salts are not as readily absorbed or biologically active as organic forms of chromium. Under certain ecological conditions yeasts are able to accumulate chromium and other trace elements and incorporate them into organic compounds. Effect of elevated chromium concentration of 3, 5, 10 and 20 mM Cr³⁺ (chromium chloride hexahidrate), and time of addition to the medium on yeast growth and accumulation of total chromium in yeast biomass were evaluated. Chromium was added in chemically defined medium in the beginning of the bioprocess (3, 5 and 10 mM Cr³⁺) and in the middle of exponential growth (3, 5, 10 and 20 mM Cr³⁺) of yeast *Candida intermedia* ZIM 156. Yeast were cultivated in batch mode in 3.5 l stirred bioreactor (V_e=2.5 l, 200 rpm, 1 vvm, 28 °C, 10% inoculum). Parameters pH, pO₂ and yeast growth were on-line measured, while accumulation of total chromium in yeast biomass was determined in different periods of time. The results showed that the level of chromium accumulation was dependent on both ambient concentration and time of chromium addition into the

medium. It was found that elevated concentration of Cr(III) in the environment caused a reduction of growth rate and biomass production but increased total chromium accumulation in yeast biomass. Chromium addition in the middle of the exponential growth phase had no notably effect on yeast growth, which was comparable with yeast cultivation without chromium addition.

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Enhancement of productivity of α -galactosidase by optimization of medium composition

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One promising methodology for improvement in fermentation technology is the optimization of media composition to enhance productivity or reduce expenses. *Thermomyces lanuginosus* is a thermophilic fungus, which produces numerous extracellular enzymes such as amylases, xylanases, invertase, lipase, proteases and galactosidases etc. We studied the production of α -galactosidase enzyme of *Thermomyces lanuginosus* CBS 395.62/b strain. We aimed to increase the α -galactosidase activity by optimization of medium composition. The effects of the carbon and nitrogen sources were investigated. The function between the enzyme activity and the concentration of raffinose as sole carbon source, moreover the effect of nitrogen source as a quality factor was examined. The used nitrogen substrates were yeast extract and L-asparagine. Their quantities were defined according to the equal nitrogen content. Application of different organic nitrogen sources did not show any significant influence on α -galactosidase activity, but the increase of raffinose concentration from 1.5 (w/v) % to 2.5 (w/v) % gave about 2 times higher activity. Taking into consideration that raffinose is an expensive carbohydrate we did not want to increase its concentration above 2.5 (w/v) %. Seven different compounds as possible nitrogen source were tested. The highest α -galactosidase activity was detected when cultivation was carried out on ammonium-acetate. To search a cheaper carbon source, fermentation was made applying various carbohydrates. The produced α -galactosidase activity was the same on raffinose and sucrose. This value was 50 IU/ml. To enhance the productivity of α -galactosidase from *Thermomyces lanuginosus* two-level factorial experiment design was carried out. Variables were sucrose [1 (w/v) %; 3 (w/v) %] and ammonium-acetate [0.3 (w/v) % and 0.6 (w/v) %]. For the evaluation of the results $Y=a_0+a_1x_1+a_2x_2+a_{12}x_1x_2$ equation was used, where Y is the α -galactosidase activity, x_1 =sucrose concentration, x_2 =the concentration of ammonium-acetate, $a_0, \dots, a_{12}$ are the

constants of the function. The results were processed by means of SPSS for Windows software. The increase of both sucrose and ammonium-acetate led to the improvement of the productivity of α -galactosidase. In this way 100 IU/ml activity was reached. Further enhancement of the productivity can be predicted by increase of both carbon and nitrogen sources.

On the base of our results it is possible to establish an industrially acceptable fermentation, where molasses can serve as main carbon source.

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Analysis of genetic variability within the *Aspergillus viridinutans* species

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The intraspecific variability of the *Aspergillus viridinutans* species was examined using various techniques including morphological examinations, carbon source utilization tests, restriction enzyme analysis of the mitochondrial and nuclear DNA, and sequence analysis of part of the β -tubulin gene. Although the genetic distances between the isolates were higher than between e.g. *A. fumigatus* and *Neosartorya fischeri*, most *A. viridinutans* isolates together with *N. aureola* and *N. udagawae* strains were found to belong to a single cluster based on sequence data. Strain FRR 1266, which was earlier classified as a highly divergent *A. fumigatus* isolate, was found to belong to the *A. viridinutans* species. The ochratoxin A producing *A. viridinutans* strain IMI 306135 was most closely related to an asexual isolate. These two latter strains were more closely related to *A. fumigatus* and *N. fischeri* than to any *A. viridinutans* strains, and possibly represent a new species in *Aspergillus* section *Fumigati*. The dendrogram based on carbon source utilization data and results of restriction analysis of the mitochondrial and nuclear DNA of the strains supported most of the evolutionary relationships observed based on sequence data. The results indicate that the presence or absence of nodding conidial heads is not an unequivocal morphological character for the identification of species within *Aspergillus* section *Fumigati*.

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K. RIGÓ, J. VARGA, B. TÓTH

Evolutionary relationships within *Aspergillus* section *Circumdati*

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Sequences of the intergenic transcribed spacer regions and the 5.8 S rRNA gene of type or neotype strains of species currently assigned to *Aspergillus* section *Circumdati* were analysed. Parsimony analysis of sequence data indicated that *Aspergillus* section *Circumdati* is non-monophyletic. *Aspergillus campestris*, *A. lanosus*, and *A. dimorphicus* with *A. sepultus* were found to be more closely related to *Aspergillus* sections *Candidi*, *Flavi* and *Cremeri*, respectively. These results were also supported by phenotypic data. *A. robustus* and *A. ochraceoroseus* were found not to be related to any of the species examined. Species of the proposed revised *Aspergillus* section *Circumdati* formed two main clades, which could also be distinguished based on phenotypic methods. Ochratoxin producing species were scattered on the cladogram indicating that ochratoxin producing ability of the strains was lost (or gained), several times during the evolution of *Aspergillus* section *Circumdati*. Ochratoxin producing abilities of the isolates examined did not correlate with their taxonomic relationships based on sequence data, although a clade found not to produce ochratoxins during an earlier study of genetic variability of *A. ochraceus* could also be identified in this study.

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Heavy metal tolerance of *Trichoderma* fungi isolated at different adaptation periods in a long-term field-experiment

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The anthropogenic heavy metal pollution of soil (e.g. field application of sewage sludge) may alter the composition of soil microbiota (Bíró 1999, Kecskés et al. 1997), resulting in a reduced natural biocontrol of the soil-borne plant pathogens. *Trichoderma* fungi are world-widely spread common soil microorganisms, which play an important role in the biocontrol processes (Cook 1993). Heavy metal tolerance of

some *Trichoderma* isolates were studied *in vitro* for the spore germination and the mycelial growth in liquid and solid medium. Strains were originating from a long-term field-experiment, where the calcareous chernozem soil was artificially contaminated by 13 heavy metal salts in four levels (0, 30, 90 and 270 mg kg⁻¹). Samples of the Cd, Ni and Zn were selected after 3 and 5 years of pollution, respectively.

Regarding the heavy metal sensitivity significant differences were found among the *Trichoderma* isolates obtained in the same year from different treatments (application rates and types of the metals). Much less differences were observed, however among the isolates, which were originating from the same metal treatments at two consecutive, different isolation periods.

Less significant changes in the sensitivity are concluded (within a two-year-adaptation periods), comparing to the high variability of metals and their application rates. The effect of heavy metal salts among the natural environmental conditions is especially complex and influenced by several biotic and abiotic environmental factors. Long-term field experiment with the separated rates of several toxic elements (in Nagyhörösök, Hungary) serve as a model background for studying the main soil-biological processes and microbial interactions (Kádár 1995).

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L. SÁMI, T. EMRI, Z. VARECZA, T. PUSZTAHELYI, I. PÓCSI

Fragmentation, autolysis and cryptic growth in ageing *Penicillium chrysogenum* cultures

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The pseudotrisaccharide allosamidin, which is known to be one of the most specific and effective inhibitors of chitinases, retarded significantly the fragmentation of hyphae in ageing carbon-depleted cultures of an industrial β -lactam-producing *Penicillium chrysogenum* strain, especially between 60 and 80 h incubation times. Under antibiotic producing conditions, allosamidin did not have any effect on either the growth or the penicillin yields. At 115 h cultivation, fragmentation was complete, even

with allosamidin treatment, giving a very similar morphology comparing to control cultures, with the dominance of yeast-like surviving forms usually consisting of two cells. Not only the number and germinating ability of these cells, but the extracellular autolytic enzyme, e.g. hexosaminidase and chitinase activities were the same. Moreover, the intracellular glutathione level, which is an indicator of the physiological state of the cells, was not disturbed by allosamidin either. These results suggest that the rate of autolysis, although shifted in time, could be very similar in the cultures.

Allosamidin also hindered, even after addition of an extra dose of glucose, the outgrowth of new tips from the surviving hyphal fragments. In spite of this, only a slight difference between the glucose utilisation rates of allosamidin-treated and control cultures was found, which means that the treated cells still had considerable metabolic activity. Moreover, after transferring the cells into fresh, glucose-containing and allosamidin-free culture medium, the treated cells regained their ability to produce new tips, showing the same outgrowth as the control cells did.

In addition to morphological changes, allosamidin also had a significant effect on the dry weight of the cells, which increased about 25%. This suggests significant changes in the structure and chemical composition of the cell wall. According to nitrogen elemental analysis of alkali insoluble wall residues, the chitin content of the wall increased considerably.

The most active extracellular chitinases, determined by SDS-PAGE were found to be 21 and 33 kDa. The properties of these enzymes are now examined.

To sum it up, the morphological effects of allosamidin on ageing *P. chrysogenum* gave a clear evidence of the involvement of extracellular chitinases in the age-related fragmentation of the fungus. Moreover, allosamidin-sensitive chitinase(s) have also been shown to play a crucial role in the outgrowth of new hyphal tips from dormant hyphal fragments, which may therefore contribute to the maintenance of the cryptic growth observed under carbon starvation. On the other hand, allosamidin did not influence the process of autolysis of this *Penicillium* strain during the examined cultivation time.

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**Analysis of the morphology of *Acremonium chrysogenum*
under producing circumstances**

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Growth of mycelial microorganisms and their interactions with the environment in the fermenter are complex. Morphology and differentiation are very important reflections of the organism physiology, which change during the course of fermentations as well as with operating conditions. Computer-driven image analysis is a vital tool to gather data on these aspects of physiology, and to relate them to fermentation conditions.

For the classification of mycelial morphology by image analysis, cells are considered as either dispersed forms or pellets. Dispersed forms are further divided into two groups, namely freely dispersed forms and mycelial clumps. The latter ones are loose aggregates of hyphae, which are not sufficiently tightly packed to be described as pellets, but which do not show a simple mycelial tree shape. Rate of conversion of the clumps onto freely dispersed forms is a good indicator of the fragmentation of mycelia.

The cephalosporin C producer *Acremonium chrysogenum* ATCC 46117 did not exhibit pellets during the course of fermentation. Proportion of clumps, roughly 60% after inoculation, stayed on this level during the first 24 hours of cultivation, then started to decline steadily, reaching a value of less than 10% of all mycelia and indicating a considerable rate of fragmentation. In contrast, the average number of hyphal tips increased from 4.5 to 6 in the first day of cultivation, followed by a heavy decline which resulted in an average tip number of 3. Similar time-course pattern was found over the average total hyphal length of the freely dispersed forms. By the 48th hour of cultivation, almost all the carbon sources had been consumed, resulting in a cease in the biomass and CO₂ production rates.

Addition of glucose quickly restored biomass and CO₂ production, and resulted in a sharp increase in the proportion of clumps, up to the starter value of 60% of all mycelia. Average hyphal area, average hyphal length and the average number of tips also increased considerably. However, as the additional carbon source was exhausted, all these changes turned into negative in a surprisingly short period of time. A renewed addition of glucose resulted in changes of the same pattern.

However, addition of soybean oil, considered as an "unregulated" and hence beneficial carbon source with respect to cephalosporin C production, failed to stimulate the above-detailed changes in morphology. Ratio of clumps to all mycelial forms decreased to low level, together with the average hyphal area, average hyphal length and the average number of hyphal tips, as well as the biomass production rate. Nevertheless, cephalosporin C production rate, negligible upon glucose addition, was boosted.

Our results clearly indicate that morphological parameters obtained by image analysis are consistent primarily with the growth pattern of the fungus. It can also be concluded, that the ratio of mycelial clumps to the total hyphal pool is the most sensitive indicator of the fragmentation process during fermentation.

T. SIMONICS, A. MARÁZ

High frequency electro-transformation for *Schizosaccharomyces pombe*

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High frequency transformation is a prerequisite for efficient transformation of cells with a genomic library. In the yeast *Schizosaccharomyces pombe*, the alkali cation method is considered as a simple and effective transformation method (Okazaki et al. 1991). We could not, however, adapt this method for our strain (S 18-82), which originated from the crossing and meiotic segregation of a transformable (D 18) and a selenate-resistant auxotrophic strain (0-82).

The aim of our experiments was to develop an efficient, high frequency electroporation method for transformation of S 18-82 *ura4* strain with genomic bank based on pUR18N vector.

We applied treatment of cells before electroporation (Gene Pulser, BioRad) with disulphide reducing agents 2-mercapto-ethanol or dithiothreitol and partly digested the cell wall with lysing enzyme (Sigma). Different parameters for the electroporation were used (voltage, capacitance and resistance) and the effect of osmotic stabilization of cells after electric pulse was also tested. By the use of optimum electroporation parameters, 10^3 and 10^4 transformants/ μ g DNA was achieved for S 18-82 and D 18, respectively.

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**Isolation of low-molecular-weight chromium species from yeast
*Candida intermedia***

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In 1988 chromium was recognized as an essential element for humans (IPCS, 1988) and adequate daily dietary intake (ESADDI) was established by the FOOD and Nutrition Board of the National Academy of Science (Food and Nutrient Board, 1989). The chromium story began in 1959 though, when Schwarz and Mertz found that chromium-rich brewer's yeast was the most efficient regulator of glucose tolerance in rats compared to inorganic chromium(III) compounds. Chromium species was named as Glucose Tolerance Factor (GTF) and Cr(III) was recognized as biologically active component of the substance GTF (Schwarz and Mertz, 1959). Ever since researchers tried to isolate the chromium GTF from yeast *Saccharomyces cerevisiae*, since it was proved that *S. cerevisiae* yields the largest quantity of naturally occurring chromium (Toepfer et al., 1973). Today, the enigma of isolated chromium binding compounds from yeasts is characterization of chromium ligands. The hypothesis is that chromium is coordinated to amino acids such as glycine, glutamic acid, cysteine or maybe to nicotinic acid which was later seriously questioned (Sumrall and Vincent, 1997). The first partial characterization of LMWCr compound was done by Yamamoto (Yamamoto et al., 1987), who isolated LMWCr from the rabbit liver (later also from other mammals) and it is believed to be naturally-occurring, biologically active, chromium-containing oligopeptide of ca 1500 daltons in mammals (Davis and Vincent, 1997). In order to contribute to chromium story, we isolated LMWCr species from chromium(III) chloride enriched yeasts *Candida intermedia* in phase of exponential growth. Biomass was harvested from two different bioprocesses, in one chromium(III) (in form of chromium(III) chloride hexahydrate) supplementation was 5 mM and in the other 20 mM. LMWCr species from yeast *Candida intermedia* were isolated as ethanol precipitates from cytoplasm. In comparison LMWCr species were isolated from biomass *Candida intermedia*, which was not enriched with chromium(III) chloride. The following comparison of all three LMWCr species from *Candida intermedia* was made by ion-exchange chromatography on Pharmacia Biotech DEAE-Sepharose fast flow support and on BIA CIM-DEAE disks. Electrothermal atomic absorption spectrometry (EAATS) and FAAS were employed for detection of isolated and separated LMWCr species. Results revealed that although total chromium content was highest in LMWCr species isolated from biomass *Candida intermedia* enriched with 20 mM Cr(III), total

LMWCr species amount was lower compared to the total amount of LMWCr species from 5 mM Cr(III) enriched biomass *Candida intermedia*. IR spectroscopy was employed to detect Cr-species in each isolation step. Size-exclusion chromatography on Pharmacia Biotech Superdex support revealed that all LMWCr species have molecular weight below 10000.

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A. SZABÓ

A comparative study of alkaline ponds in the Hortobágy and Kiskunság National Parks

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Protozoological research of small alkaline bodies may arouse interest because these biotopes are unique in Europe, or all over the world.

Small alkaline ponds (Zab-szék, Fehér-szék, Kelemen-szék, Bödi-szék) near Szeged and Kecskemét (KNP – Kiskunság National Park) evolved on sodic “solonchak” soils with alkaline upper A-layer. Therefore their water is very alkaline (pH: 9.2–9.5) all the year round, the conductivity is high (1000–9700 $\mu\text{S}/\text{cm}$) with a $\text{Na-HCO}_3\text{-Cl}$ character. These water, have a stable ion content and are usually opalescent. Their depth is 20–50 cm.

Small water bodies of Zám-puszta (south of Faluvéghalom, HNP – Hortobágy National Park) accumulating in the microgeomorphologically deeper, barren areas are found on different subtypes of “solonetz” soils with acidic A-layer, thus their water is usually neutral or slightly alkaline (pH: 7.3–8.6). Conductivity values are relatively low (470–900 $\mu\text{S}/\text{cm}$), becoming only higher (tip to 2000–5800 $\mu\text{S}/\text{cm}$) during the concentration process. These waters are opalescent, their depth is 10–25 cm. The water character is mainly Na-Cl-HCO_3 , sometimes $\text{Na-HCO}_3\text{-Cl}$.

As a consequence of these facts there are significant differences in the Ciliata fauna of the two studied areas. This can be essentially explained by the influence of the different chemical character of the waters and their effect on the species diversity.

As it has been proven by earlier studies 30–32% of Ciliata species in the small water bodies of the KNP were preferring alkaline environment while this figure in the case of the HNP ponds was found to be only 18–20%.

There are many cosmopolitan species of wide ecological tolerance spectrum in both areas. The number of species is low in the small alkaline ponds of the two parks (4–6 species as an average), but especially low in those of the KNP. It is remarkable that *Astylozoon vagans*, *Epistylis digitalis*, *Marituja pelagica*, *Hypotrichidium tisiae*, *Pseudochilodonopsis algivora* was found exclusively in the HNP ponds, while *Mycterotrix ovata*, *Pseudochilodonopsis piscatoris*, *Prorodon marinus* and *Coleps minor* only in the KNP ones.

As a result of our studies a number of species preferring definitely the alkaline, sodic environment were found even in small water bodies of Cl⁻ character, like *Epistylis halophila*, *Epistylis microdiscum*, *Uronema marinum*; *Monodinium* sp., *Hemiphrys fusidens*, *Oxitricha sirnilis*, *Vorticella difficilis*, *Hastatella radians*, *Astylozoon fallax*, *Astylozoon vagans*, *Frontonia leucas*, *Frontonia atra*.

ZS. SZILÁGYI¹, Á. GRALLERT¹, E. ZILÁHI¹, M. SIPICZKI^{1,2}

The *Schizosaccharomyces pombe sep10* gene encodes a conservative protein that presumably plays a role in transcription of a subset of genes

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Regulation of gene expression is remarkably important for unicellular and multicellular eukaryotes.

Based on scientific achievements of recent years, it is accepted that eukaryotic gene expression is an extremely sophisticated and highly organized event, which requires a large number of gene products assembled in different transcription complexes. These complexes have to communicate with activators and repressors to regulate transcription of a single gene, and consist of RNA polymerase II, mediators and co-factors, general and specific transcription factors. Therefore these factors and mediators play an important role in regulating transcription of a subset of genes, and influence numerous cell functions in context with their specificity.

Yeast is a widely used model for understanding cell processes, so that a number of putative mediator and transcription factor genes were identified from *Saccharomyces cerevisiae*, but little is known about their function. In many aspects, the fission yeast

Schizosaccharomyces pombe is more similar to higher eukaryotes and provides a powerful model, but genes have not been isolated.

By using the fission yeast, *sep* mutants with complex defects (including cell separation and mitotic abnormalities) were isolated, and eleven novel *sep* genes were identified. Three of them were cloned. *sep9* encodes a homologue of SPT8, a protein involved in the SAGA complex existing in baker's yeast, and play a role in chromatin remodelling prior to transcription. The *sep15* gene product is a homologue of the *S. cerevisiae* MED8, a protein required to mediate transcription.

The *sep10* protein shows high homology to the SOHI protein and to its homologues from *Drosophila melanogaster*, *C. elegans* and *Homo sapiens*. The *SOHI* gene is a suppressor of HPRI, which product is a member of a transcription complex, and is believed to act as an effector of several external stimuli.

The lecture will cover mainly the results of studies with the *sep10* gene, and the probable function of the protein in cell separation and differentiation will be discussed.

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Importance, detection, and taxonomic classification of human caliciviruses

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Human caliciviruses (HuCVs), members of the family *Caliciviridae*, are the major cause of acute viral gastroenteritis cases and epidemics in all age-groups worldwide. In countries where investigations for the detection of HuCVs have been done, they were found in 30 to 40% of gastroenteritis cases. These viruses spread by feco-oral route (via contaminated food, water or direct contact) without predominant seasonality, however, a small peak in the number of cases can be observed in winter and spring months under the temperate climate. In addition to sporadic cases, the outbreaks occurring mainly in semi-closed populations, such as nurseries, schools, summer-camps, hospitals, barracks, hotels, restaurants, etc., have special epidemiological importance.

Up to now nobody has been able to propagate HuCVs either in experimental animals or tissue cultures and the infections can be verified only by molecular methods on genomic level. In addition to the reverse transcription-polymerase chain reaction (RT-PCR) using fecal samples (less frequently water and food), sequencing and

subsequent phylogenetic analysis revealed the circulation of high number of strains and proved their heterogeneity, as well.

Due to the limited data of antigen-based investigations, the taxonomic classification is based on the genom (polymerase and/or capsid region) analysis. Currently, HuCVs are subdivided into two generas as Norwalk-like viruses and Sapporo-like viruses, including two (GI, GII) and one (GIII) genotypes, respectively. Within the genotypes several clades or clusters are described according to the results of comparative investigations of the individual viruses found in different geographical locations.

Because CVs, considering their importance, are still less known in our country, the aim of this presentation – as an introduction of the following accompanying lecture on the first Hungarian HuCV investigations – was to review the increasing epidemiological importance and research of these viruses, and provide information about the diagnostic development for the detection of HuCV infections in Hungary.

P. G. TORNAI-LEHOCZKI

Importance of “CHROMagar Candida” medium in differentiation of foodborne, food spoilage and opportunistic pathogenic yeasts

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CHROMagar Candida medium is a differential culture medium, which was originally developed for selective isolation and presumptive identification of some clinically important yeast species. On CHROMagar Candida medium *Candida albicans* forms green colonies, *C. tropicalis* blue-gray colonies with dark brownish purple halo, *C. krusei*, (*Issatchenkia orientalis*) pink colonies with white edge, *C. glabrata* deep pink or purple colonies. Together with the differences in colony surface this medium is capable for presumptive identification of the yeast species most commonly isolated from clinical material (Odds and Bernaerts, 1994). The reliability of CHROMagar for the above-mentioned purposes was confirmed by other authors as well (e.g. Freydiere and Gille, 1994a and 1994b).

The purpose of our work was to study the capacity of this medium for visual discrimination of yeast species frequently isolated from different kinds of foodstuffs and some opportunistic pathogenic yeast species associated with food. 190 strains, including 72 type strains, were investigated. The performance of the medium was

studied by streaking the strains on CHROMagar Candida medium. After 48–72 h incubation at 30 °C, the morphology and colour of the colonies were noted.

The investigated strains gave green, blue, blue-green, pink, deep violet, claret coloured, yellow, beige, brownish and white coloured colonies. In agreement with the earlier findings (Freydiere and Gille, 1994a) the appearance of the colonies of the investigated *Trichosporon cutaneum* strains were blue-green, but on the basis of their different colony morphology it was possible to distinguish them from *Candida albicans* and *C. tropicalis* strains. However the broadening of the spectrum of the investigated species resulted in the detection of other green or blue-green colony colours, e.g. in case of *Candida zeylanoides*, *C. sake*, *Debaryomyces polymorphus*, *D. maramus* and some *Cryptococcus* species. The colour of the colonies of the different strains representing the same species was the same or very similar, indicating that the medium is suitable for typing the strains of non clinical yeasts. In some cases the medium proved to be useful in rapid differentiation of related species pairs belonging to the same genera as well. CHROMagar Candida medium provides a fast possibility to separate *Zygosaccharomyces bailii* from *Z. rouxii*, *Debaryomyces hansenii* from *D. polymorphus* as well as *Rhodotorula glutinis* from *R. mucilaginosa*.

S. TÓTH¹, B. FILIPIČ², F. SOMOGYVÁRI³, K. KOVÁCS¹, R. KOREN²

Inhibition of cell culture growth by short impulses of ionic current, electric and magnetic fields

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Adherent cell cultures of different species origin were treated with short (10 ms) impulses of direct ionic current, were submitted to electric field or to magnetic field for the same period. The field force was 100 V/cm for the ionic current, 1000 V/cm for the electric field and 10 mT for the magnetic field. The energy densities were 1.8×10^5 J/m³, 1.1 J/m³ and 6 J/m³, respectively. All cultures showed a marked growth inhibition upon three consecutive impulses applied before seeding of the cells. Although cumulative population doubling curves run parallel – indicating a single modulation event – the effect could be observed at least for 72 hours. The greatest inhibition was observed when electric field was used. Although the culture conditions would greatly influence the growth responses of the tested cell lines to the electromagnetic stimuli, it was proven that the responses could be attributed to the cells and not to the

environmental conditions. It is also apparent that the effectivities of the three stimulus types were markedly different. The extremely high efficiency of electric and magnetic fields in comparison to direct current underlines the importance of careful assessment of the environmental risk of the electromagnetic pollution.

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**Effects of different ointments with *Saccharomyces cerevisiae*
in regeneration of epidermis**

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We studied the effect of inactivated baker's yeast for external use on rabbits. We eliminated the epidermis from exterior members of the legs, making 4 lesions with 1 cm diameters after depilation. We prepared three ointments with inactivated baker's yeast and we treated the rabbits divided in to three groups.

All animals had 4 lesions. The first lesion from left anterior leg was not treated. It was the control lesion. The second lesion from left posterior leg was treated with 5% inactivated yeast incorporated in one of the base material of ointments. The third lesion from right anterior leg was treated with one of the base material of ointments and the fourth with inactivated powder of yeast. We treated the rabbits with these preparations one hour after the epidermis elimination.

The first group of rabbits was treated with 5% inactivated baker's yeast incorporated in unguentum simplex, with unguentum simplex and with powder of inactivated yeast. The second group of rabbits was treated with 5% inactivated baker's yeast incorporated in Carbopol gel, with Carbopol gel and with powder of inactivated yeast. The third group of animals was treated with 5% inactivated baker's yeast incorporated in unguentum emulsificans, with unguentum emulsificans and with powder of inactivated yeast.

After 2 days of treatments the epitelisation process was faster on lesions treated with inactivated yeast powder and with 5% inactivated yeast incorporated in Carbopol gel. After 4 days in this regions the lesions was softer and smaller. After 6 days this sloughs was very small and very soft.

The scar process was faster on lesions treated with inactivated yeast powder, followed by lesions treated with 5% inactivated yeast incorporated in Carbopol gel. When we used the 5% inactivated yeast in base material of ointments in the first group of animals the wounds was normal and in the third group these were big and cracked. The lesions treated with base material of ointments healed later than the untreated lesions (control lesions).

J. VARGA¹, L. FERENCZY²**Taxonomic position of the cyanobacterium causing toxic algal blooms in lake Balaton**

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A filamentous cyanobacterium responsible for toxic algal blooms in Lake Balaton was isolated and cultivated in our laboratory. Morphological examination of the isolate led to the assumption that it belongs to the species *Cylindrospemopsis raciborskii*. To prove this hypothesis, nucleic acids were isolated from the isolate, and sequence analysis of the 16 S rRNA gene was carried out. A simple nucleic acid isolation protocol was developed for the isolation of high molecular weight DNA and RNA simultaneously from filamentous cyanobacteria. Plasmids or phage nucleic acids were not observed in the total nucleic acid preparations. A 1500 bp fragment of the 16 S rRNA gene was amplified and sequenced using appropriate primers. Altogether 1423 nucleotides were involved in the phylogenetic analysis. Parsimony and neighbour-joining analyses of sequence data indicated that this isolate is most closely related to *C. raciborskii* strains, although the sequence of its 16 S rRNA gene differs significantly (3.85%) from those of Australian *C. raciborskii* isolates. More *C. raciborskii* isolates of diverse origin should be examined to clarify the taxonomic identity of this strain.

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J. VARGA¹, B. TÓTH¹, SZ. SZENCZ¹, J. MOLNÁR¹, CS. FEKETE², L. SZABÓ²**Mycoviruses in *Aspergillus* species**

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Mycoviruses with double-stranded RNA (dsRNA) genomes are frequently encountered in *Aspergillus* isolates. A detailed study of such dsRNA elements in black *Aspergillus* isolates collected worldwide was carried out, and the data were analysed. The results indicate that about 10% of black *Aspergilli* are infected. However, the geographic distribution of infected isolates exhibit large variations; 3–13% of the isolates collected from different continents were found to carry dsRNA elements.

Hybridization experiments indicated that electrophoretic banding patterns are not reliable tools for estimating the diversity of these mycovirus genomes. Among strains representing other *Aspergillus* sections, dsRNA segments indicative of mycovirus infection were observed for the first time in 3 *Aspergillus* species (*A. leporis*, *A. petrakii*, *A. primulina*). The last species is able to reproduce sexually, its teleomorph is *Neosartorya primulina*. This is the second report on the detection of naturally-occurring dsRNAs in sexually reproducing *Aspergillus* species. The presence of virus-like particles in these and other *Aspergilli* was also examined by electron microscopy. Most infected *Aspergillus* isolates examined were found to carry virus-like particles in the size range of 36–40 nm.

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Genetic variability of *Rhizopus* strains assessed by random amplified polymorphic DNA analysis and carbon source utilization tests

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RAPD analysis and carbon source utilization tests were carried out on 25 *Rhizopus* isolates. A high level of intra- and interspecific genetic variability was detected. However, 3 species groups (*Rh. stolonifer*, *Rh. circinans* and *Rh. oryzae*) could be identified unambiguously. The *Rh. circinans* was originally described as an independent species, but the recent taxonomic revision of the genus (carried out on a morphological basis) did not distinguish it from *Rh. stolonifer*. Our results do not support this approach, but clearly show the need to handle *Rh. circinans* as a separate species. Strains of *Rh. oryzae* form a relatively uniform group; both the patterns of the carbon source utilization and the RAPD amplification products were very similar even for isolates originating from widely-separated places (e.g. from different continents). These data disprove the view that *Rh. oryzae* is a heterogeneous group in the *Rhizopus* genus. The mainly morphological characters, used in previous taxonomic works on this genus, exhibit too much variation to form the basis for a long-standing taxonomy and/or a safe species delimitation. Accordingly, in our work physiological and RAPD

markers were determined which are suitable for identification of the isolates of the investigated *Rhizopus* species.

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Sulphate-reducing bacteria from the healthy and the degrading reed rhizosphere of lake Velencei

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Rhizome of healthy and degrading reed stands and sediment samples from Lake Velencei were taken in June 1999. To estimate the CFU number of the sulphate-reducing bacteria (SRB) in the rhizosphere and sediment the MPN technique was used. Cultivation of the SRB took place in an anaerobic cabinet using different SRB media and finally 7 pure cultures were obtained.

The 16S rRNA gene of these strains was subjected to ARDRA and two groups have been detected. The partial sequencing of the 16S rDNA of each group was carried out.

The Anaerobic BIOLOG identification system was applied to investigate the sole-carbon-source utilization patterns of the two sequenced bacterial strains.

The number of SRB in the rhizosphere was about two orders higher than in the sediment. This can be due to the increased amount of root exudates, organic matter from the biodegradation processes and fermentation products in the rhizosphere.

The bacterial strain isolated from the rhizosphere of the healthy reed stand proved to be *Desulfovibrio alcoholivorans* at a similarity value of 98.4%. The other strain isolated from the degrading stand was closest to *Desulfovibrio fructosivorans* (95.6%) based on partial sequencing, however, when the full sequence of the 16S rDNA was analysed it was found to be nearly identical (99%) with the sequence of an environmental *Desulfovibrio* clone obtained from a French wine distillery's anaerobic digester.

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The influence of indigenous wine killer yeast on white wine fermentations – microbiological, chemical and sensorial evaluation

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The problem of top quality wine production is tightly connected with wine yeast selection and strain improvement. Selected yeast strains can provide better control over the fermentation process. The use of a selected yeast strain can improve wine quality and stability in various dimensions. For these reasons, yeast selection is a common practice in winemaking industry. General problem of traditional selections is that they are often too much oriented in the selection of an universal wine yeast strain. Our research was focussed at the selection of indigenous wine killer yeast with beneficial properties for the product, which should keep the traditional local characteristics of wine. Primary selection was done on total wine yeast population of 462 isolates. We performed selection of potential starter culture among several indigenous wine killer yeast strains of *Saccharomyces* genus, which were characterized regarding killer activity and plasmid profiles. Four carefully selected strains were inoculated in the must of Malvazija, which was prepared according to traditional standardized technology. Fermentation process and its dynamic were followed microbiologically and chemically during the fermentation time course, and sensorically as well. With microbiological assays we trace killer character of isolated yeast generated from the starter culture in four consecutive sampling points from fermentors. Further the proportion of killer starter vs. natural flora was estimated by karyotyping. It was found that indigenous flora was really suppressed by killer inoculum. With numerous chemical assays we followed accumulation of amino acids, production of ethanol and higher alcohols, conversion of sugars, level of organic acids as well as traditional enological parameters what gave an excellent view on yeast from product point of view. Since chemical status of wine is not sufficient for final decision on its quality, a testing panel was performed as well. Must transformation to wine was followed sensorically with an expert, however after five months of maturation the produced wine was evaluated according to the official method of OIV with highly skilled persons. It is encouraging to conclude that one of the selected wine killer strains produced the most favourable wine compared to spontaneous fermentation process and some industrial starter cultures from the market tested in the same Malvazija must.

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Antimycotic sensitivity/resistance of *Candida albicans* and nonalbicans species

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There are several mechanisms defending the human being against infections of yeasts colonizing the mucosa and skin surfaces of the human body or environmental vectors (equipment, food, ...), however, minor defect of the immunosystem is enough for infection caused by the most virulent *C. albicans* strains.

The higher lack of immunodefense can lead to severe invasive mycoses and can give opportunity for infections by the other less virulent fungal species.

The spectrum of the pathogen yeasts has changed and extended because of the increased number of neutropenic patients, parenteral feeding, using of catheters, etc., and usage of the (sometimes excessive) azole antifungal therapy.

While in the 1970' and 1980' the consideration was focussed on the *C. tropicalis* and *C. parapsilosis*, in the 1990' the fluconazole resistant *C. krusei* and *C. glabrata* has become most important.

There are emerging new tasks in the mycological diagnostics for exact identification and determination of the antimycotic sensitivity/resistance of the isolated strains because of the changing of the spectrum of the pathogen species.

We have tested the antimycotic fluconazol (Flu), itraconazol (Itr), amphotericin B (AmB), ketoconazol (Ket), miconazol (Mic), nystatin (Nys)) sensitivity/resistance of the most common *Candida albicans* strains (296) and "nonalbicans" *Candida* strains (144): *Candida colliculosa* (1), *C. glabrata* (70), *C. guilliermondii* (1), *C. inconspicua* (5), *C. kefyr* (6), *C. krusei* (15), *C. lipolytica* (1), *C. lusitaniae* (7), *C. magnoliae* (1), *C. norvegensis* (10), *C. parapsilosis* (12), *C. tropicalis* (10), and *Geotrichum candidum* (1), *G. sp.* (1), *Rhodotorula sp.* (3), *Saccharomyces cerevisiae* (16), *Trichosporon beigelii* (4), *T. capitatum* (3), *T. sp.* (1) by Etest, Fungitest and disk diffusion in 1999–2000 (I–II quarter)

While the large amount of the *Candida albicans* strains was proved to be sensitive or intermediate sensitive to the tested antimycotics other yeast species (*Candida glabrata*, *Candida krusei*, *Candida lusitaniae*, *Candida norvegensis*, *Candida parapsilosis*, *Candida tropicalis*, *Saccharomyces cerevisiae*) seemed to be resistant in higher rate in case of some antimycotics (Flu, Itr, Ket, AmB). The development of resistance against nystatin, miconazole, however, was not found important.

Summary:

1. A revision of the list of – mainly fluconazole – resistant *Candida* species must be made.
2. The identification and discrimination of the biochemically similar *Candida* species should be made very carefully, as test kits commercially available might cause some problems.
3. The problematics of monitoring, therapy and profilaxis should be reconsidered because of the increased number of Amphotericin B resistant isolates (mainly in case of *C. krusei*).

BACTERIOLOGY AND VIROLOGY

S. G. B. AMYES

Genetic problems of penicillin-resistant pneumococci

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Streptococcus pneumoniae is a common cause of severe community-acquired pneumonia. For many decades, this pathogen has been treated successfully with penicillin and until recently, has remained sensitive. However, within the last decade, there has been a steady increase in penicillin resistance; which has, in some areas, resulted in the more than 50% of isolates losing their sensitivity. This decrease in susceptibility is manifested by changes in the penicillin binding proteins, particularly by the formation of mosaic genes. The acquisition of penicillin-resistance is accompanied by a corresponding increase to most of the beta-lactam antibiotics, including the carbapenems. Interestingly, the proportional increase to cephalosporin resistance is higher raising the question whether their usage has promoted the selection of beta-lactam resistance. The bacteria that become resistant to beta-lactams are also likely to become resistant to the macrolides, suggesting that these strains have a pre-disposition towards the acquisition of resistance.

These bacteria do not, however, have an innate capability to become resistant to the fluoroquinolones. There are a number of new fluoroquinolones currently targeted at pneumococci. Resistance can emerge to them but at a relatively low frequency. There is some debate as to which is the main target of these new fluoroquinolones, and thus which mutations contribute most to resistance. The traditional view is that the main target is Topoisomerase IV, but there is increasing evidence to suggest that the Gyrase A subunit is, as it is in Gram-negative bacteria, the primary target of the fluoroquinolones and that mutations in this protein are the major cause of resistance.

A major difficulty in following the epidemiology of resistance has been in the typing of the bacteria. They are traditionally typed by serology; however, more recent genotyping techniques do show considerable variation within a single serotype suggesting that it may be difficult to identify clonal spread, and thus epidemic formation, if phenotyping is used alone.

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**Oxacillinase activity in clinical isolates of borderline methicillin resistant
*Staphylococcus aureus***

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Staphylococcus aureus is one of the most threatening Gram-positive clinical pathogen, which with increased β -lactam resistance can cause many therapeutical failures. At first the penicillin resistance, then soon after the introduction of methicillin the appearance of methicillin resistant was described. Methicillin resistance is termed intrinsic due to the production of an additional, low affinity penicillin-binding protein. Recently, *S. aureus* strains with borderline methicillin resistance have been identified. Borderline phenotype cannot be attributed solely to the classical penicillinase, since a second, methicillin hydrolysing enzyme (methicillinase) is produced.

The aim of our work was to search borderline methicillin resistance strains among clinical isolates of *S. aureus* in order to study the resistance mechanism.

We have worked with altogether 53 clinical isolates and according to their MIC values four were found to be borderline resistant. In the presence of β -lactamase inhibitors (clavulanic acid, sulbactam) their MIC values to oxacillin were reduced. To determine the enzyme activity, we used whole cells as enzyme sources. We measured the β -lactamase activity with and without methicillin induction. All the four strains produced inducible β -lactamases. The induction ratios (the ratios of the β -lactamase activities of the induced and uninduced cells) were between 12 and 24. For typing β -lactamases we used β -lactamase reference strains. According to the activities of the cefazolin/cephaloridine and nitrocefin/cefazolin ratio, the β -lactamases produced by the four strains proved to be type A.

For the measurement of the hydrolysis of oxacillin we elaborated two different methods. In the first one, the hydrolysis of oxacillin was determined spectrophotometrically. Nitrocefin was applied as control compound. The oxacillin was hydrolysed by the cells in detectable extent. The whole-cell preparation contained both the extracellular and membrane-bound β -lactamases. To clarify whether the extracellular or the membrane-bound enzyme was responsible for the hydrolysis of oxacillin, we isolated extracellular enzymes and purified by affinity chromatography. These preparations did not hydrolyse oxacillin in detectable extent. In the second method, membranes were prepared in order to demonstrate that oxacillinase activity originated from the membrane fraction. The isolated membranes were preincubated with oxacillin solution, and the residual concentrations of oxacillin were measured by agar diffusion method using *Bacillus subtilis* test strain. The isolated membranes were

able to split oxacillin. Our observations confirmed the findings that a membrane-bound oxacillinase is responsible for the borderline methicillin resistance in *S. aureus* strains.

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**Nucleic acid investigations of the acute bee paralysis virus
and development of a diagnostic PCR assay**

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An unusual adult bee and brood mortality was observed in some colonies of an apiary near Budapest. In healthy honey bee pupae infected by an injection of bacteria free extract of the diseased adult bees a virus was detected by electron microscopy, and the virus was later identified as acute paralysis virus (APV) by serological investigations.

The isolate was propagated by intra-abdominal injection of white- or light brown-eyed pupae from symptomless colonies. The pupae were maintained at 35 °C for three days and were homogenized afterwards. Viruses were purified from the suspension and concentrated by caesium-chloride gradient ultracentrifugation. The viral RNA was isolated and cDNA was synthesized by oligo (dT) primer method. The cDNA was amplified using virus-specific primers and primers designed under conserved sequences of related picornaviruses. The polymerase chain reaction (PCR) provided specific products. A PCR product was sequenced and aligned to other sequences available at genebank database. The highest consensus was found with a sequence fragment of an APV strain isolated in the United Kingdom. Now we are concerned with sequencing further parts of the genome.

Viral nucleic acid was detected directly by RT-PCR from some bee samples from apiaries of five counties of Hungary. We would like to survey the distribution of the acute paralysis virus in the Hungarian apiaries using RT-PCR. We would also like to proof the presence of other bee pathogen viruses by propagation in pupae and electron microscopic investigations.

Our research was supported by OTKA C077 and T030335 projects.

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Effect of simulated microgravity on human lymphocytes

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During space flight the function of the immune system changes significantly. This is partly due to microgravity condition itself. We wished to analyse mechanisms responsible for changing leukocyte functions in low gravity environment. For terrestrial simulation of microgravity we used a Rotary Cell Culture equipment developed by NASA.

We investigated the effect of simulated microgravity on separated human peripheral blood lymphocytes. Different cell populations were detected with the help of Flow Cytometer (Coulter EpicsXL) by antibodies conjugated to fluorescent dyes (anti CD4-PE, anti CD8-PE and anti CD19-PC5). Since the microgravity during space flight reduces the number of peripheral lymphocytes we supposed that apoptotic (programmed cell death) processes might be induced. This hypothesis was supported by our earlier result demonstrating that simulated microgravity increased the level of Tumor Necrosis Factor-alpha (a known apoptotic signal molecule) significantly.

For the detection of apoptosis we applied the Annexin-V propidium-iodide Assay which is based on the appearance of phosphatidil-serine (PS) in the outer leaflet of the membrane. PS is recognized by FITC conjugated Annexin-V protein. As propidium-iodide cannot penetrate through intact membranes, this DNA binding fluorescent dye discriminates between cells with apoptotic PS distribution and disrupted cells (caused by other mechanisms) with PS in the inner leaflet of membrane.

During a three days period of observation simulated microgravity did not affect the percentage of apoptotic cells in T helper (CD4+) or cytotoxic T (CD8+) cell populations. On the contrary, the percentage of B cells (CD 19+) dying by programmed cell death showed a significant increase over control.

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The effect of mutant Rab 5 endosomal proteins on adenoviral viropexis

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The function and the material circulation of the early endosomal compartments that play an important role in adenoviral viropexis are affected by different protein

containing systems. The function of these proteins is often essentially important. The examination of mutants of these proteins gives important details on the interaction between the virus and the host cell.

Our former investigations proved that the inactivated epsilon COP protein is able to partially inhibit the virus penetration to the cell. As Rab 5 proteins similarly play a basic role in the endosomal material circulation, in this study we examined the effect of these proteins on the adenoviral viropexis: Our data suggest that the mutant Rab 5 N133I with dominant negative effect surprisingly increases the viral entry, although it inhibits the fluid phase endocytosis in the other hand. We presume that the change in the dynamics of the endosomal material circulation stands in the background.

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Family-carriage of a *Staphylococcus aureus* strain examined by classical and molecular microbiology methods

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Staphylococcus aureus strain isolated from a chronic recidival furuncle and its family spread was examined. The strain was isolated from furunculous wound of a 16-year-old girl and also from nose or hand samples of her parents and of two sisters. Biotyping by STAPHYtest 16, BBL Crystal Gram positive ID and ATB ID Staph panels, antibiograms, phage patterns, pulsed field gel electrophoretograms and AP-PCR patterns suggest the presence of the same clone within the family.

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Investigation of human rotavirus strains in six consecutive rotavirus seasons in Hungary

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Human rotaviruses are the major etiologic agents of viral acute gastroenteritis in childhood. Based on differences of the two surface antigens (G and P antigens, respectively) many serotypes have been described. The authors aimed to provide data of strain diversity, temporal and geographical distribution of human rotaviruses circulating in two regions (Budapest including the metropolitan area and Baranya county) of Hungary. 1240 rotavirus positive stool samples collected in six consecutive seasons (1994/1995 to 1999/2000) were tested with MAb-EIA. The majority of the samples represented the four most common serotypes (G1 to G4) as follows: G1- 61.3%, G2- 14.5%, G3- 1.5%, G4- 1.9%. Mixed infections were detected in 3.2% of the samples. 9.4% of the specimens were non-typeable, partly due to strong cross-reactions. Furthermore, 8.2% of the samples showed reactivity with the VP7 specific common MAb (MAb 60), but not with any of the serotype specific MAbs. Some of these (0.9% of the total) displayed G6 specificity and represented the first isolation of this serotype in Hungary and its second detection in Europe. Results obtained with MAb-EIA were confirmed using RT-PCR (144 of 1240) and nucleic acid sequencing. These surveys were extended to the P antigen, as well. In addition to the most common strains (P[8]G1, P[4]G2, P[8]G3, P[8]G4) five additional genotype combinations were identified (P[6]G4, P[9]G3, P[8]G9, P[9]G6, P[14]G6). Although similar strain diversity has been previously demonstrated, those reports were mostly from tropical settings.

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Induction of full replication cycle of human cytomegalovirus by the Tax protein in CD4+ T cells

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Human cytomegalovirus (HCMV) is one of the most frequent opportunistic agents causing severe illness in chronic human T cell leukemia-lymphoma type I (HTLV-I) infection. Our previous studies have shown that coinfection of macrophages with HCMV and HTLV-I significantly enhances HCMV replication. HCMV release from dually infected macrophages alone, however, could not account for symptomatic HCMV infection in patients suffering from adult T cell leukemia (ATL). HCMV has been shown to latently infect CD4+ T cells. We hypothesized that interaction between HCMV and HTLV-I in ATL cells was also critical to the dissemination of opportunistic HCMV infection. For this purpose, we studied HCMV replication in various T cell lines. In HUT-78 and H9 cells no replication of HCMV was observed. In contrast, HCMV infection of HTLV-I-carrying MT-2, MT-4 and C8166-45 cells resulted in release of infectious progeny virus. Since cellular transcription factors NF- κ B, CREB and SRF play a crucial role in HCMV replication, presence of these Tax-inducible proteins was investigated in the various T cell lines by electrophoretic mobility shift assay. Results showed high levels of NF- κ B, CREB and SRF expression in MT-2, MT-4 and C8166-45 cells, whereas none of these transcription factors were found in HUT-78 and H9 cells. Using an inducible system (JPX-9 cells), significant NF- κ B, CREB and SRF expression as well as HCMV replication were observed. These data strongly suggest that the effect of Tax on HCMV replication in T cells is accomplished through at least three host-related transcription factor pathways.

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Characterization of tight-binder peptide ligands to *Bacillus subtilis* spores

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Phage display is a novel method for analysing protein-protein interactions. Obviously, it might be also helpful to study the spore coat assembly in *Bacillus subtilis*, which relies on the proper interaction of several structural proteins and enzymes.

We used an M13 phage display library expressing random heptamer peptides to select ligands, which bind tightly to *Bacillus subtilis* spores. After several rounds of biopanning we enriched for heptamers containing an NHFLP consensus sequence at the N-terminus of the peptides. We constructed two phages expressing the tetramer sequence NHFL and a pentamer sequence NHFLP. Using competitive biopannings we were able to show that NHFLP sequence is also a sufficient binder but not the tetramer NHFL sequence.

Doing sequence similarity search we found the NHFLP sequence close to the N-terminus of the SpsC protein. Its N-terminus is the following: MVKQRNHFLPYSLPL... SpsC protein is responsible for spore coat polysaccharide biosynthesis along with other proteins transcribed from the *spsA-K* gene cluster which knock-out makes the spore surface more hydrophobic. Based on the theory that with phage display we can mimic naturally occurring protein-protein-protein interactions we proposed that this protein is processed and needs the NHFLP sequence for binding to the spore surface. We constructed several phage expressing decamer sequences from the SpsC N-terminus: MVQQRNHFLP, RNHFLPYSLP and NHFLPYSLPL. We analysed phage binding to *Bacillus subtilis* spores with flow cytometry and only the NHFLPYSLPL phage gave a fluorescent shift. This result supports the protein processing theory. Expressing wild type and N-terminal deletion mutants of SpsC and doing further flow cytometry on *Bacillus subtilis* spores would completely confirm our proposal.

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Influence of chestnut tannins on growth of *Clostridium perfringens* and *E. coli*

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Introduction: Chestnut tannins are a mixture of hydrolysable and condensed tannins, known to react nonspecifically with proteins, forming insoluble complexes. The result of this interaction is an inactivation of the proteins' biological activity. *Clostridium perfringens* type C is the etiologic agent of haemorrhagic enterotoxaemia in piglets, lambs, calves, foals and sheep and necrotic enteritis in chickens. *Escherichia coli* causes different conditions affecting intestine, toxemia and sepsis in many domestic animals and men.

Materials and methods: As a source of tannins we used Farmatan (Tanin, Sevnica), containing 70% of tannins. The influence of tannins on the growth of *Clostridium perfringens* type C (ATCC 51880) was tested in Brain heart infusion broth. After 12 hours anaerobic incubation at 37 °C tenfold dilutions were prepared from which cfus on blood agar plates incubated anaerobically for 24 hours were counted and numbers of *C. perfringens* calculated. In the preliminary test concentrations of 0.5% and 0.05% tannins were used. Twofold dilutions down to 0.0015% were used to determine MIC. *E. coli* G 491 (0138;K81,88ac) was tested with the concentrations of 2.5%, 0.5% and 0.05% of tannins in peptone water (PW), Brain Heart Infusion Broth (BHI) and Trypticase Soy Broth (TSB). The inoculum was 2.2×10^6 cfu/ml. After an aerobic incubation of 8 hours at 37 °C, cfus from twofold dilutions on blood agar were counted and numbers of *E. coli* calculated.

Results and discussion: Within 12 hours 0.05% tannin in BHI reduced an inoculum of 4.4×10^4 cfu/ml of *C. perfringens* more than 44-fold (i.e. under detection limit), while the number in BHI increased 382-fold. Concentrations of 0.025% and 0.0125% reduced numbers of *C. perfringens* in inoculum of 5.4×10^4 cfu/ml of HBI more than 54-fold, concentration of 0.006% allowed only a slight growth to 7.3×10^4 , while concentrations of 0.0031% and 0.00625% reduced growth only 2.7-fold and 1.7-fold, respectively, compared to 9.3×10^7 cfu/ml in BHI.

The best growth of *E. coli* was found in TSB with an 244-fold increase of inoculum, followed by 188-fold in BHI and 98-fold in PW. Only a small reduction of growth was observed in media with 0.05% of tannins. In media with 0.5% of tannins the inoculum increased only 2-fold in PW, 5-fold in BHI and 46-fold in TSB. The concentration of 2.5% of tannins reduced inoculum more than 2180-fold (under detection limit) in PW, 436-fold in BHI and 218-fold in TSB. Comparing both bacteria

C. perfringens proved to be much more sensitive to tannins than *E. coli*. In *E. coli* the sensitivity was greatly influenced by growth medium like BHI and PW versus TSB.

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Evaluation of prebiotics in different microbial system

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In the past few years science has discovered the importance of intestinal microbiota and the role of functional food in maintaining its natural, healthy balance. Functional food contains pre-, pro- and symbiotics thus exerting positive effect on the human body.

Probiotic food additives consist of living bacterial culture (*Bifidobacterium*, *Lactobacillus*). When they get into the body they serve as substrates of the intestinal microbiota and have a positive influence on it. There are short chain fatty acids among the metabolic products of these bacteria that have an antibacterial effect by lowering the pH of the colon. They also improve the state of the immune system and help to re-establish the intestinal microbiota following an antibacterial treatment. Prebiotics serve as nutrients for probiotic bacteria. Prebiotics are non-living food components that go through the intestinal tract undigested and act as substrates for intestinal bacteria. Some oligosaccharides have prebiotic, bifidogenic effect. Human digesting enzymes cannot break down these oligosaccharides, thus blood sugar level does not rise, but they help digestion and the growth of beneficial intestinal bacteria.

The aim of the work was to investigate which commercially available prebiotic oligosaccharides are able to support the growth of bifidobacteria. Enteropathogenic bacteria were also taken into consideration as secondary selection factors. It was found that all commercially available prebiotics can serve as nutrients for bifidobacteria. The enteropathogenic bacteria utilise fructo-oligosaccharides and some of the isomalto-oligosaccharides. The only exception was a product called Oligotime that does not support the growth of the investigated pathogens (*Salmonella derby*, *Salmonella choleraesuis*, *Shigella dysenteriae*, *Shigella flexneri*, *E. coli* O55H6, *E. coli*, *Proteus mirabilis* and *Yersinia enterocolitica*). Oligotime is an isomalto-oligosaccharide in which 90% of the glycolytic bonds are $\alpha(1-6)$ ones.

As a result of our investigation it is possible to create a symbiotic functional product that contains bifidobacteria as probiotics, and prebiotics as well to sustain their viability while not supporting pathogenic bacteria.

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**Is there any importance of the detection of "high-risk" HPV types
in Gynecological cervical screening program?**

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Patients: 100 women were examined, who potentially suffer from "high-risk" HPV infection, the diagnosis was raised by the pathologist screening of the cervical smear, or by the gynaecologist screening the surface of the cervix by colposcopy

Methods: Samples were taken by Digene special kit from the surface and from the canal of the cervix, which contained epithelial cells. DNAs were isolated and using "high-risk" HPV specific primers (MP09-MP11 primer pair, Research Genetics) were amplified by PCR, high-risk-specific oligonucleotides were shown by agarose electrophoresis.

Results: The study shows that the possibility of "high-risk" HPV type infection is likely at women, whose cervical smear are suspect, or colposcopic atypia was detected. We directed our attention to those cases, where "high-risk" HPV infection was detected without cytological or colposcopic abnormality.

Zs. CSUKÁS¹, M. BAUSZ², J. TÓTH², I. SÜVEGES², F. ROZGONYI¹

**Bacterial and mycological examinations of the corneal discs removed
with keratoplastica**

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At the 1st Department of Ophthalmology of Semmelweis University 73 corneae removed by keratoplastic surgery between 01 September 1999 and 30 April 2000 were investigated with histological, bacteriological and mycological methods.

Indications of keratoplastica most frequently included degeneration of corneal endothelium, dystrophy as well as ulcers of corneae.

Out of 73 specimens tested 29 bacteria were isolated including 3 aerobic and 26 anaerobic strains. No fungi were isolated. Most frequent isolates included *Propionibacterium* *ances* (11), *Peptostreptococcus* spp. (6), anaerobic *Corynebacterium* (3), *Stomatococcus* (3), *Gemella* (1) and *Clostridium bifermentans* (1).

Antibiotic susceptibility as well as their enzyme and toxin production associated with their pathogenic role were investigated in *Propionibacterium* strains.

A. CZEGLÉDI, E. FERENCZI, E. TÓTH, I. MEZEY, GY. BERENCSEI

Serosurvey of tick-borne encephalitis virus (TBEV) in Hungary

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Sera of healthy individuals older than 20 years of age were tested for TBEV antibodies. All sera were tested by ELISA and those of borderline or slight positive result were confirmed by immunofluorescence (IFA) and haemagglutination inhibition test (HAI). Altogether 129 positive samples were identified of 2256 sera following verification (5.72%).

These specific antibodies may be produced either following natural infection or due to active immunisation with one of the killed vaccines available.

The seropositivity of 107 serum samples could not be verified using the alternative techniques (4.7%). The origin of false positivity could not be identified yet. Previous experiments suggested that the association of false-positivity and inappropriate collection or storage of serum samples was not significant. TBE seropositive persons were found to be present in all areas of Hungary. As determined earlier in connection with the diagnostic screening of clinical diseases the majority of the seropositive inhabitants are concentrated in 5 forested counties. The seroprevalence was found to be 13% in Baranya, 12% in Nógrád, 10% in Vas, 8% in Zala and only 6% in Somogy. 90% of the total number of seropositive individuals lived in the above 5 counties.

Based on our recent results and the earlier field trials in natural foci the Transdanubian region and the Northern central chain of mountains are endemic territories. The distribution of seropositive sera was: 36% in the Transdanubian ridge of hills, 22% in the Plain in Northwestern Hungary including the Subalpine region, 19% at the Northern central chain of mountains, 13% at the Transdanubian central chain of mountains, whilst only 10% in the inhabitants of the Great Hungarian Plain. The data

of Budapest and Pest county were omitted, since these two administrative areas are located at the border of three different geographical regions.

The age group between 20 to 29 years possessed the highest seroprevalence. Thus they belong to the age group of highest risk and/or this population takes advantage of the vaccination.

The rate of antibody positive persons was found to decrease with the age. One can suppose that a significant proportion of seropositive samples originates from vaccination.

Significant deviation was found in comparison to the results of earlier investigations in the distribution of positive samples by gender. It was found earlier that men represent a higher proportion (70%) of persons having antibodies against TBEV. According to the results of this study, the difference between the two genders was reduced from 70 to 54%, but the dominance of men still exists. The shift is probably caused by the post-vaccination seropositivity of the population examined.

J. DEÁK

Laboratory diagnosis of *Bartonella henselae* and *Bartonella quintana* infections

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Of the currently known 11 *Bartonella* species 5 can cause different human illnesses. Cats, bobcats, mountain lions and captive wild felids are the reservoirs of *Bartonella henselae* (*B. henselae*); the vector is the flea. *B. henselae* may well be the most frequent cause of chronic lymphadenopathy at a young age. The lymph node lesions may be local or generalized. *B. henselae* has been detected in cases of Parinaud oculoglandular syndrome, neuroretinitis, osteomyelitis, fever of unknown origin, prolonged fever and inoculation peliosis, and as etiological agent in the acute encephalopathy of 7 children within some weeks in Florida; 62% of cats (n=124) living near the homes of these children revealed antibodies against *B. henselae*. *Bartonella quintana* (*B. quintana*) was earlier known as present in trench fever. Humans acquire the bacterium via transmission by lice. *B. quintana* is responsible for bacillary angiomatosis, endocarditis and meningoencephalitis. The bacterium has been detected in cases of subcutaneous and bone lesions and peliosis. *B. quintana* infections are frequent among homeless and alcoholic people who have lice. The agent can cause vascular proliferative diseases in HIV-infected patients.

Between 1 September, 1998 and 10 May 2000, we carried out tests for the detection of *B. henselae* and *B. quintana* IgM and IgG antibodies by an indirect immunofluorescent method. The clinical samples of the patients predominantly revealed lymphadenopathy.

B. henselae IgM and IgG were detected in 10.3% (n=291) and 31.9% (n=338), and *B. quintana* IgM and IgG in 4.8% (n=289) and 12.6% (n=340) of the patients. *B. henselae* and *B. quintana* antibodies were detected simultaneously in 15% (n=289) of the cases.

The distribution of *B. henselae* and *B. quintana* antibodies varies in different human and feline populations throughout the world. 100% of patients who had suffered cat scratch disease in Germany exhibited antibodies. 29.9% of cat owners in Spain displayed *B. henselae*-specific antibodies, as compared with only 5.9% of the control blood donors. Cats have *B. henselae*-specific antibodies between 9.1% and 68.0%. *B. quintana* antibodies were detected in 30.0% of the serum samples of homeless persons in Marseille, in 20.0% of those in Seattle, and in 12.8% of the inhabitants of a refugee camp in Burundi. At the same time, the largest epidemic of typhus caused by *Rickettsia prowazekii* since World War II was likewise observed in Burundi. *B. henselae* infections were detected serologically in a higher percentage than *B. quintana* in Hungary. Cross-reactions are presumed in some cases between *B. henselae* and *B. quintana*. The expansion of these almost forgotten and newly discovered bacterial agents may be connected with ecological and economic changes.

G. DIÓSI, L. GAZSÓ, GY. FARKAS

Study of biofilm development in nuclear waste repositories

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Budapest, Hungary

Six dominant strains were isolated from the interim storage of the spent fuel at the Atomic Energy Research Institute. The metabolism of the isolates and their effects on biocorrosion and heavy metal mobilization were studied. The sulphate reducing capability and the siderophore productivity of the isolates were low, but we found the organic acid productivities positive and also the level of the toxic metal uptake (Cd, Cr, Co, Sr) was high. These properties show the possible danger of corrosion and metal mobilization. The radiosensitivity of the isolates was determined. The D_{10} values of the spore-formers were ranging between 1.44–1.88 kGy. We checked the biofilm formation and the micromorphology of the biofilm development. In order to make a

useful model of the microbial effects on the materials at the storage of the spent fuel it is not enough to examine the metabolism of bacterial cells in suspension. The biofilm is the dominant form practically in all realistic systems, that is why it is important to obtain information about the metabolism of the biofilms and its effects on different metal surfaces. We plan to examine the level of the toxic metal uptake by the biofilms and to compare the radiosensitivity of the biofilm bacteria with that of the cell suspensions in the cases of pure and mixed cultures.

GY. FARKAS, L. G. GAZSÓ, G. DIÓSI

Characterization of subterranean bacteria in the Upper Permian Siltstone Formation

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The main purpose of this work was to study the microbiology of the Hungarian Upper Permian Siltstone (Aleurolite) Formation from the aspect of the safety of future underground repositories for nuclear waste. Air, groundwater, technical water, rock and surface samples (n=67) were collected aseptically from different depths. The number of aerobic and anaerobic isolates was 300. The gases produced by the 16 gas forming isolates were CO₂ (aerobic isolates) and CO₂ and H₂ (anaerobic isolates). About 20% of the aerobic isolates produced siderophores. The proportions of organic acid producers were lowest in aerobic and anaerobic isolates from the aleurolite: 13% and 14%, respectively. The highest proportions of acid producers in the aerobic and anaerobic isolates from the air samples were 63% and 54%. Altogether 160 of the aerobic isolates and 52 of the anaerobic isolates were sporeformers. The radiosensitivity of the aerobic and anaerobic isolates was also determined: the D₁₀ values of the aerobic spore-formers were ranging between 0.8–2.44 kGy, and those of the anaerobic spore-formers were 1.86–4.93 kGy. Our results indicate that the sulphate-reducing bacteria and the production of complexing agents (siderophores) may contribute to the mobilization of radionuclides from underground repositories. As well, microbial gas production can influence the environmental conditions. The variability in bacterial radiotolerance indicates the biodiversity at this potential disposal site. These facts must be considered during the planning of a nuclear waste repository.

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Studies on competitive growth between *Lactococcus lactis* and *Listeria monocytogenes*

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In the frame of the EU CT97-3129 (PREMIUM)-project, growth of a *luxAB* bioluminescent transformant of *Listeria monocytogenes* in co-cultures with an "acid only" *Lactococcus lactis* strain was studied in their co-cultures in OXOID Brain Heart Broth supplemented with 0.3% yeast extract and 0.2% additional glucose concentration, and adjusted to various pH and NaCl concentrations. Besides liquid cultures, gelified cultures containing 12% of low melting-point gelatine were also studied in order to mimic the growth conditions in gelled parts of foods. In various experiments, the effect of the initial pH, incubation temperature, and salt concentration were investigated, together with the effect of the relative proportion of the initial levels of the lactic acid bacteria and the *Listeria*, on the outcome of the competition.

In the course of incubation the changes of the viable cell counts by plate counting in selective media were estimated from time-to-time, together with the measurement of pH and the titratable acid contents, and the luminometric light emission of the populations of the bioluminescent transformant of *Listeria monocytogenes* was measured as well. The obtained growth curves were evaluated for growth parameters by fitting growth data with Baranyi's "D-model".

The light output of the bioluminescent *Listeria* cultures increased parallel with their viable cell counts during the exponential phase of the growth. At the commencement of the stationary phase of the growth, the bioluminescence activity dropped significantly.

In experiments, where *Lactococcus lactis* was able to compete with the *Listeria*, the initial growth rate of the target organism was not influenced, but an early induction of its stationary phase occurred resulting in lower maximal population densities than those during its monoculture growth. The induction of the early stationary phase in co-cultures coincided always with a sudden drop of pH (and thereby a sudden increase of the concentration of the undissociated lactic acid formed in the cultures).

The competitiveness of *Lactococcus lactis* has been influenced by the salt concentration of the medium. In experiments with an initial pH of 7.0 and 15 °C incubation temperature, *Lactococcus lactis* was more sensitive to the increase of the NaCl concentration in the growth medium than *Listeria monocytogenes*, because the specific growth rate values of the latter remained within the same confidence interval in

the whole concentration range of 0.5 to 4.0% investigated, while 4% NaCl slowed down so much the growth of *Lactococcus lactis* that it was unable to induce an early stationary state in *Listeria monocytogenes*.

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Plasmid characterization and replicon typing of verotoxic and enterotoxigenic *Escherichia coli* carrying F18 fimbria

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The F18 fimbria are characteristic adhesive antigens of porcine enterotoxigenic *E. coli* (ETEC) and of verotoxigenic *E. coli* (VTEC). During the last few years the phenotypic and genotypic characterization of F18⁺ ETEC and VTEC strains have been done by us in several respects but data about the exact localization of the F18 genes and about their possible plasmids are still needed.

Therefore we have performed investigations on Hungarian F18⁺ strains (17 ETEC and 7 VTEC) and concluded that the F18 genes were consequently on their plasmid compartment and that these plasmids belong to the F incompatibility group.

Our methods were: DNA probe fragment preparation by PCR from the reference strain F107/86 and plasmid preparation from the strains to be tested by the method of Kado-Liu. Further DNA hybridization on the plasmids was done by using the following ³²P labelled replicon probes: repFIA, repFIB, repFIC, repF2A, repFI1.

The ETEC and VTEC strains investigated all possessed 1–4 plasmids (above 40 kb). The F18 genes were localized on one of these large plasmids, with the exception of one VTEC strain (2206), which proved to be free from large plasmid and free from F18 genes.

The replicon typing of the plasmids indicated that they belonged to the incompatibility complex F and represented the type FIC or one of its subgroups FI1A or FI1. The plasmids tested had only one replication origin (unireplicon). Further studies on the F18 plasmids are in progress.

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**Novel serum replacement based on bovine ocular fluid: a useful tool
for cultivation of animal cells *in vivo***

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Different mammalian cells in culture have individual nutritional requirements, which are mainly fulfilled by the addition of fetal calf serum to the basic medium. Due to the batch to batch variability and relatively high price different serum replacements were introduced. Among them bovine colostrum as a serum substitute for the cultivation of mouse hybridomas should be mentioned. The presented experiments were aimed to introduce the simple and effective serum replacement (SR) based on bovine ocular fluid. Throughout the experiments bovine ocular fluid alone and in combination with sheep's defibrinated plasma and serum albumin was tested for growth of different cells growing as monolayer: (a) cell lines: HeLa, WISH, HAC-3/T2, HAC-3/T3 (human amniotic cell lines) WiREF (Wistar rat embryonal fibroblasts) MDBK (bovine kidney), PLA (adult pig kidney), ST (swine testis) (b) primary culture: chicken embryonal fibroblasts. All the growth experiments were made in parallel with Fetal Calf Serum (FCS). All types of cells were cultivated in usual Eagle's medium + antibiotics (Penicilline, Streptomycine, Gentamycine). The most effective was the SR containing approximately 35% of sheep's defibrinated plasma and 1.5% of serum albumin in bovine ocular fluid. During the experiments 1, 5 and 1% of SR or FCS in Eagle's medium were used. After 2, 3 and 5 days of cultivation, the cells were counted. The results show that the use of SR instead of FCS gives higher number of cells. It is also important that practically no adaptation is needed, meaning that the cells could be grown in the Eagle's medium + FCS and in the next passage in Eagle's medium + SR and *vice versa*.

A. FÜZY¹, ZS. LANGÓ¹, E. TÓTH¹, L. G. TÓTH², I. MUSKÓ²

**Bacteriological data on selected molluscs and planktonic crustaceans
of Lake Balaton**

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The molluscs and the planktonic crustaceans are filter-feeding organisms and some of them are considered to be effective bacterial feeders. The aim of our work was to identify the bacterial communities of the hindgut content of two molluscs (*Anodonta* sp., *Unio* sp.), the gut content of the *Dreissena* and *Corophium* sp., and homogenized body of *Bosmina*, *Daphnia* and *Eudiaptomus* sp. We collected three species of molluscs and four species of planktonic crustaceans at two sampling sites (eastern part-Tihany and western part-Keszthely) of Lake Balaton. From the seven species we isolated seventy-two representative strains and studied their morphological, physiological and biochemical characters and the strains were identified by the Biolog system, too. Based on eighty-three coded characters a clusteranalysis was performed. Dendrogram was obtained by using the UPGMA algorithm and S_{SM} coefficient with the help of the SPSS for Windows 7.5 software. Ten dominant phenons were delineated, where the cluster's overall similarity is eighty-ninety percent. The dominant clusters contain the following taxa: *Micrococcus* sp., *Bacillus cereus*, *Acinetobacter* sp., *Pseudomonas* sp., *Klebsiella* sp., *Aeromonas* sp., *Bacillus megaterium* and two other phenons, identified only on family level: Enterobacteriaceae and Vibrionaceae. These organisms are typical in the water and sediment of the lake, too. Like in case of some fishes the aerobe and facultative anaerobe microbiota of these molluscs and crustaceans can be organized from the dominant organisms of water and sediment. Seventy-nine percent of the strains were Gram negative. The typical enterobacterium *Klebsiella oxytoca* was found only in the hindgut of the bigger size of molluscs (*Anodonta* sp., *Unio* sp.). Most of the facultative anaerobe bacteria were found at the region of Keszthely.

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Serological survey on the presence of some zoonoses in European brown hares in Slovenia

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Objectives: The aim of our study was to evaluate serologically the exposure of European brown hares (*Lepus europaeus*) in Slovenia to the causative agents of some important zoonoses and consequently consider their involvement in the transmission of these diseases.

Material and methods: European brown hare sera were collected in 1999 from the hares shot in different regions of Slovenia. ELISA and/or seroagglutination (SA) tests were used to determine antibodies against *Borrelia burgdorferi* (*B. burgdorferi*) and *Francisella tularensis* (*F. tularensis*) in 73 serum samples; 48 sera were examined for the presence of antibodies against *Brucella* spp., *Toxoplasma gondii* (*T. gondii*) and Tick-borne encephalitis (TBE) virus.

Results and discussion: Antibodies against *B. burgdorferi* were found in 49.3% of sera (ELISA). 1.3% of sera were positive in ELISA using *F. tularensis* antigen whereas all the samples were negative in seroagglutination test. In 4.2% of sera antibodies against *Brucella* spp. (ELISA) were found. 41.7% of sera were positive for the presence of antibodies against *T. gondii* (SA) and 10.4% of sera against TBE-virus (ELISA).

The results indicate the importance of European Brown hares in the transmission and maintenance of Lyme borreliosis, toxoplasmosis and infection with TBE virus in natural habitat in Slovenia.

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The modification of antibacterial effect of erythromycin in the presence of dihydropyridines

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Sixteen substituted dihydropyridines of nifedipine (NP) analogues were tested on three different *E. coli* strains by Checkerboard titration. The compounds had high MIC values on the three bacterial strains. When the compound GB₁-GB₁₆ were used in

combination with erythromycin some of the compounds GB₁-GB₃, GB₄-GB₁₀ reduced the MIC values of erythromycin on the laboratory *E. coli* K12 LE 140 F[']lac strain. When the substituted dihydropyridines were tested on *E. coli* Ap^s, Er^r strain the reduction of MIC values were similar to the previous strain but not identical. The MIC values of erythromycin were reduced in the presence of GB₁-GB₇ in a lower extent on a clinical isolate of *E. coli* polyresistant strain. The GB₁₂ and GB₁₆ were the most effective in the interaction with erythromycin.

The synergistic effect of dihydropyridines depends on their chemical structure, modified by the substituents.

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Pathogenesis and pathogenicity of Malaysian isolates of infectious bursal disease virus

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Outbreak of infectious bursal disease (IBD) in poultry involving a highly pathogenic strain of serotype 1 IBD virus (IBDV) was first reported in Malaysia in 1991. Since then, the disease has become one of the most threatening diseases in poultry industry in the country. Outbreaks of IBD continue to occur in both the vaccinated and non-vaccinated flocks. The outbreaks caused great economic losses due to high mortality, impaired growth, high carcass condemnation and profound immunosuppression. Mixed infection of IBD with other viral or other pathogens are commonly reported. Evaluation on the safety and efficacy of the imported IBD vaccines available in the market commercially for local use demonstrated that most of the vaccines studied were unsafe and not effective to confer full protection against IBDV. Fourteen local isolates of IBDV from the field outbreaks in broilers, layers and village chickens were successfully isolated and propagated in chorioallantoic membrane (CAM), chicken fibroblast (CEF) tissue culture and specific pathogen free (SPF) chickens. The differences in pathogenicity of the isolates were characterized using both the conventional and molecular techniques. A highly pathogenic isolate of IBDV (UPM 94283) caused 100% mortality in SPF chickens with typical lesions of severe haemorrhages and necrosis in the bursa of Fabricius. Severe haemorrhages also occurred in the muscles and at the junction of the proventriculus and gizzard. A highly immunogenic with low in pathogenicity isolate of IBDV (UPM 93273) was attenuated in CAM and CEF tissue culture. A live attenuated IBD viral seed for vaccine development was successfully established. The viral seed (vaccine) prepared in CAM

is highly immunogenic and able to induce high level of IBD antibody when inoculated in both the SPF and commercial chickens. The vaccine is also able to neutralize a high level of maternally derived antibody (MDA). It is safe and effective to confer full protection against IBDV when given in chickens either for a single vaccination at day 14 of age or double vaccination at days 14 and 28 of age either through intraocular or oral route of vaccination or as food based vaccine. The vaccine is also safe and effective when used for *in ovo* vaccination at 18-day-old embryonated chicken egg. Studies on the pathogenicity and immunogenicity of the local IBD viral seed prepared in CEF tissue culture showed unsatisfactory result. Studies on the pathogenesis of the disease following oral route of IBDV inoculation demonstrated that the virus initially replicates in the lymphoid cells in the intestinal tract leading to primary viraemia and replication of the virus in the bursa of Fabricius, the target organ for IBDV. The virus can also enter into the bursa directly through the intestinal lumen. Localization and replication of IBDV in the bursa caused severe bursal necrosis and secondary viraemia leading to destruction of other lymphoid organs, especially the thymus and spleen. The virus was initially replicating in the Harderian gland following intraocular route of inoculation. Only a highly pathogenic strain of IBDV isolate could cause severe lesions both in the bursa of Fabricius and other lymphoid organs. In contrast, the mild pathogenic isolate produced lesions in the bursa of Fabricius with sign of bursal recovery at later stage of infection. The local IBDV isolates are also used for the development of IBD reagents and diagnostic kits and for further development of sub-unit or recombinant IBD vaccine.

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Specific enzootic dermatitis in a breeding sheep flock

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Between December 1998 and January 1999 a specific, enzootic dermatitis was observed in a crossbreed sheep flock in Northern Hungary. The affected flock comprised 400 pregnant ewes and 65 (16.2%) of them showed clinical signs during the course of the disease. In the first half of November of 1998 these animals grazed on a maize stubble-field, where the left corn-stalks were 35–40 cm high. It was likely that these corn-stalks injured the skin of the head. Two weeks later the first clinical signs could be observed.

Clinico-pathological lesions were usually restricted to one side of the head and particularly affected the skin around the eye, over the nasal and maxillary bones. Skin lesions were erythematous, swelled, and irregularly shaped ranging in diameter from 3–8 cm. Affected skin surfaces were covered by serofibrinous or serosanguinous exudate. In some cases the odour of the head was foul-smelling. The circumscribed lesions were covered by reddish-brown or brownish-black scabs and were surrounded by a zone of alopecia.

Untreated ewes showed a self-cure, their skin lesions persisted for five or six weeks and healed without scar formation. Local or parenteral antibiotic treatment was effective, the skin lesions healed within two weeks in these cases.

Histologically the following tissue lesions could be found: necrosis and desquamation of superficial layers of the epidermis, ulceration, leakage of serofibrinous exudate. Subepidermal layers of the dermis were infiltrated by mononuclear cells and neutrophil granulocytes, haemorrhages and cluster of Gram-positive bacteria could also be seen. Bacteriological culture of the scabs originated from six affected ewes yielded coagulase positive *Staphylococcus aureus* plus coagulase negative staphylococci and streptococci in three cases, while in the other cases arcanobacteria, streptococci and moraxellae grew in mixed culture. *S. aureus* strains were identified and characterized by conventional tests and commercial kits (BBL Crystal, APIStaph, Staphaurex). Ectoparasites and dermatopathogenic fungi could not be demonstrated. Blood sera of six affected ewes were negative for the presence *orf* virus antibodies, but were positive for pestivirus (BVD/BD) antibodies.

The observed clinical signs and the results of bacteriological culture were very similar to those reported by Scott and others in connection with the "Staphylococcal dermatitis of sheep" (Vet Rec 107, 452–454 1980.).

This disease has not been reported earlier in Hungary.

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**DNA sequence of a small, unidentified plasmid isolated
from a *Haemophilus somnus* strain**

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One of the plasmids present in a *Haemophilus somnus* (*H. somnus*) strain isolated from nasal discharge of a cattle with respiratory disease symptoms was purified and cloned for DNA sequencing. The copy number of the plasmid was enough to use alkaline lysis method for DNA plasmid purification which is widely used for high-copy-number cloning vectors of *E. coli*. The plasmid was found to be 1065 base pairs long and with 39.2% G + C content. Homology search failed to find any similar DNA in the nucleic sequence data bases. The longest putative open reading frame was 261 pairs long. Based on the location of possible translation initiation codons, there is a theoretical possibility on this plasmid to code for a protein of 43 amino acids. The homology search, however, showed no significant similarity with the presently available protein data.

Presence of plasmids in *H. somnus* was detected by some authors (Fussing and Wegener, 1993; Appuhamy et al., 1998) but this is the first report on the sequencing and DNA analysis of one of the plasmids.

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**Seroprevalence of humanherpesvirus-8 in Hungarian myeloma patients
and blood donors**

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The seroprevalence of KSHV in the Hungarian population and in clinical groups was determined by recombinant orfK8.1 and orf65 antigen ELISA and by anti-LANA-IFA. ELISA results were confirmed by Western blot.

In the blood donor group the IgG seroreactivity was 1.24% (6/482) to recombinant orfK8.1 antigen and 1.45% (7/482) to recombinant orf65 carboxyterminal antigen. Results were comparable with previously determined anti-LANA results 1.87% (9/482). The overall seroprevalence rate in the blood donor group was 3.3% (16/482) by these methods. The age distribution of antibodies showed a moderate but significant increase with senescence.

IgG antibodies were not detected in 31 multiple myeloma patients with any of the methods.

In a group of classical KS patients IgG antibodies were detected in 87.5% (14/16) to orfK8.1 antigen, 68.7% (11/16) to orf65 carboxyterminal antigen and 93.7% (15/16) to LANA. 9 of 16 KS patients had antibodies to all three antigens, 15 of 16 had antibodies at least to two antigens.

Our results indicate the low prevalence of KSHV in the Hungarian general population and no serological association of KSHV with multiple myeloma.

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Comparative analysis of HBV antigens and HCV antibodies using classical ELISA and commercial rapid tests

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There are more and more requests to the laboratories for the rapid diagnosis of acute viral diseases. One should exchange or replace first of all diagnostic procedures, which are time consuming, and require expensive equipments or highly specialized technicians.

There are several rapid tests approved by the FDA in the USA (Constantin, 1999). The donors of organ transplantations are prescreened for HBV, HCV, and HIV at the site of the accidents even in Hungary by rapid tests. The results of a systematic comparison are reported here. Series of serum samples pretested with HCV Antibody ELISA (Monolisa HCV Antibody manufactured by Pasteur-Sanofi) and with HBsAg ELISA (IEPANOSTIKA HBsAg ELISA ORGANONTEKNIKA) were reexamined with 4 rapid tests manufactured by 3 different firms. Two of them were specific to anti-HCV antibodies, 2 were able to detect HBsAg and HBeAg.

The ELISA results are shown in the form of OD/CUTOFF values. The results above 1.0 have been considered to be positive. The results of the rapid tests were considered to be "indeterminate", when the positive result appeared significantly later and weaker than that indicated in the manufacturers' description

	HBsAg R.T. ¹ (whole blood)	HbsAg R.T. ² (serum)	HCV R.T. ¹	HCV R.T. ²
HbsAg ELISA +	18 of 28 positives 2 indeterminates	12 of 16 positives 1 indeterminate		
HbsA ELISA –	9 of 9 negatives	4 of 4 negatives		
HCV ELISA +			14 of 23 positives 1 indeterminate	14 of 23 positives
HCV ELISA –			11 of 11 negatives	11 of 11 negatives

No false positive results were obtained. Using the rapid tests the proportion of the confirmed ELISA-positive sera found to be negative was 1:5 to 1:3 of the total number of samples tested. The OD/Cutoff ratio was between 2 to 6 of the samples found to be false negatives in the rapid test systems. In one case even a PCR-positive HCV sample was found to be negative.

The conclusion of the work is that neither diagnostic nor epidemiological studies may be based only on results of rapid tests without verification by classical ELISA and blotting or IF procedures

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Role of natural waters and water ecosystems in the epidemiology of plant viruses

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During nearly two past decades an increasing and considerable attention has been paid to the research on the occurrence of plant viruses in water ecosystems. Natural waters may play vector role in the epidemiology of plant viruses. The primary source of plant viruses in natural waters may be infected plants living near or even in the water. Besides this, water and uliginal plants – mainly perennials – may be reservoirs of economically important plant viruses. The waters of rivers and lakes are generally used for irrigation. Therefore the knowledge of the contamination of natural waters and water ecosystems with plant viruses is important from the aspect of microbiology, crop production and environmental protection.

In the last years surveys were carried out on the presence of plant viruses in natural waters and water plants in Hungary. Samples of 24 plant species were collected near Lake Balaton, Kis-Balaton and Lake Velence. 57 water and 320 plant samples

were tested by DAS-ELISA serological method for the presence of 26 plant viruses. The results of serological tests were confirmed by biological assay on herbaceous test plants and in several cases immunosorbent electron microscopy (ISEM) was also used.

According to the results out of the investigated water samples 22 proved to contain plant viruses. Cucumber mosaic virus, potato virus M and plum pox virus were found most often. Arabis mosaic virus, potato virus M, tomato spotted wilt virus, wheat dwarf virus and zucchini yellow mosaic virus were found for the first time in natural waters. On the basis of serology, cucumber mosaic and Arabis mosaic viruses were present most often in the plant samples. The proportion of the complex infection was very high, especially in case of *Mentha longifolia*, *Lycopus europaeus* and *Bolboschaenus maritimus*. No viruses were detected only in 9 species. Natural occurrence of potato virus Y and sowbane mosaic virus on *Alisma plantago-aquatica* was confirmed by immunosorbent electron microscopy as well. As the economically important viruses can occur under natural conditions in natural waters and water plants, examination of the infection chain is necessary in the future.

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First report of the circovirus caused postweaning multisystemic wasting syndrome of pigs in Hungary

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Porcine circoviruses were first recognized in cultured cells of pig origin in 1974 but they were not until 1991 associated with a characterized disease (postweaning multisystemic wasting syndrome; PMWS) in Canada. The syndrome was first observed in Hungary in December 1999. Since then PMWS has been diagnosed on five occasions.

The clinical signs occurred at the age of 40–50 days, or at a later stage, i.e. between 70–100 days of age. Retarded growth, loss in weight-gain, increased consumption of food and mortality were observed. The animals became dull, pale, anaemic, and underweighed. Additionally, dyspnea and occasionally coughing could be observed. In some cases, diarrhoea, icterus, necrotic dermatitis or abscesses in the skin were seen. The majority of the affected pigs died within two to three weeks, while some animals perished in days. The morbidity was 20–30 per cent while mortality reached 15 per cent.

The pathological investigation revealed lung edema, ascites, hydrothorax and hydropericardium. In some cases lymphadenopathy, pneumonia, mulberry heart disease, esophogastric ulcers, *E. coli*-diarrhoea, hepatodystrophy and the enlargement of the spleen were observed. The histopathological investigations showed lymphohistiocytic to granulomatous interstitial pneumonia, depletion of the lymphoid elements in the lymph nodes, and infiltration by histiocytes and the presence of giant cells. Using polymerase chain reaction porcine circovirus type II (PCV II) was detected in the bone marrow and lymph nodes originating from the thoracic cavity.

Cells negative for the presence but permissive for the cultivation of porcine circoviruses were used for the isolation of PCV II. No cytopathogenic changes were observed even after 6–8 passages, however, the cell culture supernatants proved to be PCR positive after 2–3 passages.

The decline of production of pig herds between the age of 5–13 weeks in association with anaemia, lung edema, depletion of lymphoid elements and the occurrence of giant cells indicates the presence of PCV II and, consequently PMWS. The correct diagnosis requires special virological investigations including PCR.

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Comparative studies of virulence associated genes from pathogenic and nonpathogenic *Neisseria* species

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The genus *Neisseria* includes pathogenic and commensal species. *Neisseria meningitidis* and *N. gonorrhoeae*, which cause meningitis and gonorrhoea, respectively, are the two obligate human pathogens of the genus. The commensal species exist as normal inhabitants of mucosal flora in healthy humans.

Among others pilin, outer membrane proteins, and IgA protease are known virulence factors in gonococcal and meningococcal disease.

In this study the distribution of specific sequence encoding virulence associated proteins in pathogenic and commensal *Neisseria* was investigated. First we checked the pilin expression by electronmicroscope and by monoclonal antibodies. PCR was used to detect *pilE* sequences in *Neisseria* species.

Class 1 outer membrane protein is a product of *porA* gene and expressed by most meningococcal isolates. It is responsible for increased resistance to bactericidal antibodies and can thus influence the virulence of the bacterium. PCR of the *porA* gene can give comprehensive typing or subtyping. In our study no other *Neisseria* species other than meningococci were detected by PCR.

IgA protease is an extracellular enzyme that specifically cleaves the heavy chain of human IgA. This enzyme may be an important determinant of microbial virulence. DNA slot blot hybridisation was used to determine whether a microorganism carries a particular sequence of IgA protease gene. Southern blot hybridisation was performed to further investigate the similarity. Detection of IgA protease gene by PCR suggests that sequence of this gene is present only in meningococci, demonstrating the specificity of PCR methods.

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**Antibody responses to *Chlamydia pneumoniae*, cytomegalovirus
and human heat shock protein 60 in the development of coronary heart
disease**

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Although many risk factors for atherosclerosis have been identified, the etiology of the disease is still not clear. Recent studies have suggested the role of infections and/or autoimmune mechanisms. We have investigated the antibody responses to *Chlamydia pneumoniae* (*C. pneumoniae*), cytomegalovirus-immune early proteins (CMV-IE) and human heat shock protein 60 (hHSP) in patients with coronary disease and control subjects for their independent or joint effects on the development of the disease. *C. pneumoniae* antibodies were detected by microimmunofluorescence, CMV-IE and hHSP 60 antibodies were measured by ELISA, evaluation of data was carried out by SPSS 9.0 for Windows statistical program. Antibodies to *C. pneumoniae* and hHSP 60 were present in significantly higher percentage and higher titer in patients than in control subjects, and their presence was independent from classical risk factors. There was no significant difference in the presence of CMV-IE antibodies in atherosclerotic and control groups. The high antibody levels to hHSP 60 or *C. pneumoniae* represented independent risk factors,

however the presence of both hHSP 60 and *C. pneumoniae* antibodies increased the probability of the development of atherosclerosis.

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B. KOCSIS

Old and new facts in research of bacterial lipopolysaccharides

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Endotoxin was discovered by R. Pfeiffer in 1892. During the first half of the next century methods were developed for isolation of endotoxic material from different Gram-negative bacteria: A. Boivin, O. Westphal, C. Galanos and others were successful in this field. The purified endotoxins were analysed by biochemical methods and their chemical structures were determined. An endotoxin molecule consists of a lipid and a polysaccharide component. After this discovery the endotoxin is sometimes called as lipopolysaccharide – LPS (MJ. Shear 1943). In the next period the fine structure, biosynthesis, the genetic background of biosynthesis, chemical synthesis, biological activity, clinical role and detection of LPS was extensively investigated. Nowadays for example we know that the classic enterobacterial LPS molecule contains lipid A component, which is responsible for the great number of toxic biological effects: The variable polysaccharide O-side chains give the serological specificity of Gram-negative bacteria. The core regio connects the lipid A and O-side chain to each other. The LPS molecule has numerous humoral and cellular effects: it can cause disseminated intravascular coagulation, it can activate different cells: B and T lymphocytes, monocytes, endothelial cells, it can induce the synthesis and release of cytokines: interleukin 1, tumor necrotic factor and so on. These processes are very important in development of clinical symptoms (e.g. fever, DIC, hypotonia), when a patient is suffering from Gram-negative sepsis. Now we have very sensitive methods (limulus test) for detection of LPS from different samples. Nowadays we know that some antibiotics can easily release LPS from Gram-negative microbes (e.g. cephalosporins) and others cannot (e.g. aminoglycosides). Anti-LPS monoclonal antibodies, soluble receptors, nontoxic LPS fragments are under investigation in the therapy of Gram-negative sepsis and endotoxemia. Nowadays the molecular background of the clinical symptoms, so LPS-binding proteins (LBP), LPS-receptors of different cells (e.g. CD14) and intracellular events after LPS signalling are the main scientific topics in research of bacterial lipopolysaccharides.

The bio- and immunochemical laboratory in our institute was founded by prof K. Rauss in the early fifties. The most important results from this laboratory: G. Nogrady (1920–) and M. Rodler developed a useful polytropic medium for identification of enteric bacteria (Act Microbiol Hung **1**, 437 1954), T. Kontrohr (1932–) isolated and identified 2-amino-2-deoxy-L-altruronic acid from *Shigella sonnei* phase I (Carbohydr Res **58**, 498 1977), T. Kontrohr and B. Kocsis determined the chemical structure of nucleotide sugars taking part in biosynthesis of heptose regio of bacterial lipopolysaccharide: ADP-DD-heptose (J Biol Chem **256**, 7715 1981) and ADP-LD-heptose (J Biol Chem **259**, 11858 1984). They separated these heptose nucleotide sugars by HPLC (J Chromat **354**, 417 1986) and the eight epimeric 2-aminoaldohexoses by GC (J Chromat **291**, 119 1984). Nowadays we analyse the bacterial total proteins and outer membrane proteins by capillary electrophoresis (I. Kustos, B. Kocsis, I. Kerepesi, F. Kilar: Electrophoresis **19**, 2317 1998 and Electrophoresis **19**, 2324 1998) and the serological cross-reactions between *P. morganii* and other entric bacteria (Z. Peterfi, B. Kocsis: Immunoassay in press).

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Detection of human papillomavirus-specific peripheral T lymphocytes using an IFN-gamma ELISPOT assay

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Background: The cellular immune responses to HPV are considered important for the regression of virus induced lesions. Quantitative assays of cellular immunity have not been widely used and cell responder cell frequencies and their HPV type specificity are unknown.

Methods: We investigated the peripheral blood T lymphocyte responses against purified capsids of human papillomavirus (HPV) types 6, 11, 16 and 33 in an IFN-gamma ELISPOT assay. The HPV 16-exposed group of 6 patients with cervical intraepithelial neoplasia positive for HPV16 DNA and 2 healthy HPV16-seropositive blood donors was compared with a control group of 12 healthy HPV 16-seronegative blood donors. HPV6 seropositivity was found in 6 of the 8 HPV16-exposed and in 5 of 12 control persons.

Results: The ELISPOT reactivities of the control samples revealed that detection limit of the assay was 10 responder /10⁵ lymphocytes. The responses above

the detection limit were considered positive and ranged up to 82 responder/ 10^5 lymphocytes. The HPV16-specific IFN-gamma responses were associated with HPV16 exposure (6/8 cases and 1/12 controls responding, $p=0.004$). Interestingly, the HPV33 specific responses were also significantly associated with the cases (4/8 cases and 0/12 controls responding, $p=0.014$). By contrast, the responses against the capsids of HPV types 6 and 11 were not significantly different between the HPV 16-positive patients and the HPV 16-negative controls. There was a significant correlation only between the magnitude of IFN-gamma responses to HPV6 and HPV16 (Pearson's correlation, $r=0.83$, $p<0.001$).

The corresponding HPV6-specific responder frequencies was lower than the HPV16 specific ones (linear regression coefficient, $B=0.304\pm0.048$) suggesting that HPV16 induced T lymphocytes are partially cross-reactive with HPV6.

Conclusions: HPV infection induces virus-specific T lymphocyte memory, which is readily measured in IFN-gamma ELISPOT assay. The T lymphocyte mediated immunity seems to be essentially type-restricted, although HPV6 and HPV16 may be partially cross-reactive.

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Electrophoretic analysis of outer membrane components in wild type and isogenic mutants of *Escherichia coli*

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Pathogenicity of *E. coli* strains is regulated by several virulence factors. Expression of these factors is influenced by various regulatory mechanisms, among them the global regulators have particular significance. One of the most important global regulators is the product of the *leuX* gene, which regulates the expression of α -haemolysin, fimbrial adhesins and motility.

The aim of our study was to analyze the role of this regulator in the expression of outer membrane proteins (OMP) and lipopolysaccharides (LPS). Experiments were performed by the wild type and four isogenic variants of urinary *E. coli* isolate: 536 (wild type), 536/21 (deletion mutant), 536A102 (*leuX* mutant), 536 Δ 21pSU2716 (*leuX* mutant with vector plasmid) and 536/21pGBB51 (*leuX* mutant with vector plasmid complemented with the *leuX* gene).

Examination of bacterial proteins was performed by a new method, dynamic sieving capillary electrophoresis (DSCE), which is a well applicable, suitable, automated and quantitative method in protein analysis.

In the first step of our work outer membrane protein profiles were investigated. All *E. coli* 536 variants possessed 5–6 major protein peaks in their OMP patterns. The profile of the wild type of *E. coli* 536 could be characterized by the presence of five dominating peaks, with molecular weights of 17, 19, 27, 35, and 37 kDa, respectively. In the patterns of the *leuX* mutants the absence of the 19 kDa protein, the significant decrease in the quantity of the 35 kDa protein, and the appearance of a 42 kDa peak could be observed. In the profile of the pGBB51 mutant an additional characteristic peak could be detected in the 48 kDa region.

LPS molecules were investigated by conventional SDS-PAGE and silver staining. All *E. coli* strains examined possessed a ladder pattern in the gel, characteristic for "S" bacteria, so *leuX* mutation did not influence the expression of "S" character.

Capillary electrophoresis was suitable for the investigation of protein profiles in different genetic variants. The significance of these examinations is that the outer membrane components of Gram-negative bacteria participate in several bacterial functions (pore formation, colicin and bacteriophage receptors, adhesion to the host cells, etc.). Thus changes of these factors can indicate modified permeability, pathogenicity, or colicin and phage sensitivity of bacterial cells. Further investigations are needed to elucidate how these changes in OMP profiles influence the above functions.

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Legionellosis: epidemiology, national incidence

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Legionellosis has become a considerable nosocomial disease by the end of the 90s. It is well known that *Legionella* gets into the respiratory system through the usage of industrial cooling towers, fountain, gardening houses and the hot water systems of hospitals and households (washbasins, showers etc.), with the developed contaminated aerosol. Patients who are treated with immunodeficiency in hospitals and get immunosuppressive therapy are endangered the most. By the application of modern diagnostic methods the number of legionellosis, confirmed with laboratory

investigations, has increased in Hungary. Most diagnostic methods of legionellosis are adopted successfully in the Bacteriological Department of National Center for Epidemiology (OEK) (revealing of antibodies and antigens by IF and ELISA methods, breeding). In 1999 112 blood tests out of 708 were positive. Out of 77 urine samples the antigene was revealed in 3 cases. Out of 76 BAL and sputum specimens 27 ones were positive by direct IF. In 1999 27 legionellosis were reported, which lead to death in 4 cases. Among our cases – according to the probable origin of the infection – there were nosocomial infections, diseases in connection with travelling, and occupational diseases as well. In contrast foreign data the most illnesses occurred among 16–30 year old people. The ratio of males and females (near 1:1) differs from the literary data as well. Most of our isolated strains – contrary the literary data – did not belong to *L. pneumophila* 1 serogroup and some of them proved to be ciprofloxacin resistant.

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Detection and examination of hydrogen peroxide producing lactobacilli in lower genital tract samples

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Lactobacilli are known as predominating protective microorganisms of normal vaginal flora, however, there are still unknown details of their protective microbial mechanisms against pathogens. Hydrogen peroxide produced by some strains may play an important role in control of human vaginal ecosystem, besides the low pH-value generated by main metabolites. The detection of quantity and distribution of species of H₂O₂ producing and nonproducing lactobacilli would help diagnostic efforts of bacterial infections and anaerobic vaginosis. Authors worked out a specific culture medium for isolating H₂O₂ producing lactobacilli from routine clinical samples. Selectivity of the culture medium is based on low pH-value, differentiation is associated with benzidine-reaction. Due to the chromogenic substrate H₂O₂ producing lactobacilli strains form characteristic blue colonies. Microscopically and macroscopically suspect colonies were confirmed by API 50 CHL tests.

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Actinomycosis of dogs caused by *Actinomyces hordeovulneris*

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Actinomyces hordeovulneris was isolated from the lesions of chronic pyogranulomatous pleuritis and pericarditis of one of three dogs showing similar symptoms. The parietal pleura and the pericardium were markedly thickened and covered with fine short threads of angiofibroblastic tissue. About 500–1000 ml reddish, purulent exudate accumulated in the thorax of all dogs examined containing large number of rice-sized, soft, yellowish-white granules ("sulphur granules"). When they were stained using the Gram method, a central core of branching filaments of Gram-positive bacteria embedded in thick granulation tissue could be observed. The parietal pleura, the mediastinal sheets and the pericardium were infiltrated predominantly with neutrophils, and to a lesser extent with lymphocytes and plasma cells. A small number of eosinophils and giant cells were also observed. A large number of pyogranulomas embedded in the granulation tissue were composed of a core of necrotized granulation tissue, mixed with clusters of Gram-positive branching bacteria, surrounded by an area of intact and degenerating neutrophils and lymphocytes. Bacteria were detected in the lesions by Brown-Brenn staining and they were isolated from one of the affected animals. The isolated bacteria were identified as *A. hordeovulneris*. This is the first isolation of *A. hordeovulneris* in Hungary.

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Torovirus detection in diarrheal calves and in weaned pigs in Hungary

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Toroviruses belong to the *Torovirus* genus of the *Coronaviridae* family, order *Nidovirales*. These viruses are morphologically similar and antigenically related. The bovine torovirus is an established etiological agent of disease in cattle, although the porcine torovirus was isolated from healthy animals. Evidence for the existence of toroviruses has been described in many European countries and in the United States. In

Hungary virions were detected in diarrhoeic calves by one of us (1986), which were identified as "Breda Agent" based on their morphology.

We planned a RT-PCR survey in order to detect toroviruses in Hungary. The study covered ten swine-, and nine bovine – randomly chosen – stocks. Rectal swabs and faecal specimens were collected from diarrhoeic calves and from healthy piglets after weaning. Altogether 200 porcine and 111 bovine samples were tested by RT-PCR with torovirus specific primers. We found positive specimens from calves (4) and pigs (10), respectively. In these cases we strengthened the specificity of our result by sequencing the RT-PCR product of 135 bases size.

As a result of our work we detected, first time in Hungary, toroviruses in two cattle-, and in two pig herds.

The authors would like to thank Prof. Éva Nagy and Prof. M. C. Horzinek for providing primers and positive controls.

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Nutrition-biological role of probiotic *Bifidobacteria* and their growth stimulating prebiotics

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For decades it has been well known that fermented dairy products play an important role in nutrition, because they contain living lactic acid bacteria which have beneficial effects on the host's health. Among the food products, which nowadays are on the market, there are more and more so-called functional foods, which contain probiotic, prebiotic and symbiotic additives. Probiotics are living microorganisms that upon entering the host take part in the life-process of the colonic microbiota and have positive effects on its balance. Prebiotics are not living substances but non-digestible food ingredients (e.g. oligosaccharides) that beneficially affect the host by selectively stimulating the growth of the beneficial bacteria resident in the colon. Symbiotics food contains both probiotic microorganisms and prebiotics, which selectively support the growth of the probiotic strains. In the process of screening probiotic strains attention has to be paid to several criteria. Besides technological requirements (oxygen tolerance, fermentation capability), physiological features (acid tolerance, colonisation capability) and the positive effects to the human health should not be neglected. Many producers use starter culture containing *Bifidobacteria*. Several positive effects of *Bifidobacteria* have been experimentally proved (production of vitamins, stimulation of immune

system and reduction of cholesterol level). Among the positive effects the most important one is the following: *Bifidobacteria* produce short chain fatty acids as endproducts of their metabolism acidifying the pH of the colon, thus inhibiting the growth of most of the harmful bacteria.

Our work was done in two directions. The first aim was to isolate *Bifidobacteria*, which have better features than the strains nowadays used in the dairy industry (having good adhesion capability and antagonistic effects to enteropathogenic bacteria) and to search a growth substrate, which is non-digestible and selectively stimulates their growth. The second part of our work was to test the effect of *Bifidobacteria* and different kinds of oligosaccharides on the colonic microbiota in *in vivo* animal experiments.

From our results it can be concluded that the strains isolated from human faeces have better adhesion capability and their antagonistic effects against several enteropathogenic bacteria are more effective than the type strains examined. As prebiotic the isomalto-oligosaccharide containing product was the most convenient, because this supports the growth of *Bifidobacteria* but does not support the growth of several enteropathogenic bacteria. In the experiments with mice – when they were fed functional food containing *Bifidobacteria* and oligosaccharides – we did not find any significant change in the composition of faecal microbiota. When the colonic microbiota of mice were eliminated by antibiotic treatment, they recovered after the 4 week feeding period with synbiotic food. The final goal of this project is the creation of pre-, pro- and synbiotic functional foods.

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**Mucosal and peripheral cytokine production in patients
with *Helicobacter pylori*-related duodenal ulcer**

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In *Helicobacter pylori* infection both bacterial and host factors contribute to gastroduodenal mucosal damage. Cytokines play a crucial role in the initiation and modulation of gastrointestinal inflammation. The aim of this comparative study was to investigate the mucosal production of TNF- α , IL-6 and IL-8 in patients with duodenal

ulcers, compared with the peripheral cytokine patterns. In addition we investigated the presence of antibodies against CagA and VacA virulence factors.

A considerable TNF- α , IL-6 and IL-8 expression was detected by ECL Western blot assay in both the antrum and corpus biopsy samples from 10 of the 12 *H. pylori* positive patients. On the contrary, the cytokine levels in their peripheral blood was not significantly different from that of normal healthy donors. Similarly, no significant difference concerning the *in vitro* cytokine production was observed comparing the activity of their peripheral leukocytes and that of healthy controls. All of the patients had antibodies against Cag A and Vac A antigens, as it was tested by Western blot and ELISA assays. The *in vitro* cytokine inducing capacity of different strains of *Helicobacter pylori* with and without virulence factors was also investigated.

Conclusions: Inflammatory cytokines generated locally within the gastric mucosa could be relevant to the gastric physiology of *H. pylori* infection. These results suggest a possible pathogenic role of these cytokines in *H. pylori*-associated duodenal ulcer. Further *in vitro* studies are required to determine the mechanism of cytokine induction.

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Models for reversal of resistance

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Many of the potent antibiotics have lost their effectiveness in the last decades. Antibiotic resistant bacteria are known to be responsible for more than 50% of hospital acquired infections. The multiple resistance means that patients with infections are ill for a longer time and they are at a greater risk of dying. At the same time too few drugs are developed to replace those which have lost their effectiveness. To solve these problems new compounds or combinations of compounds could be introduced to overcome drug resistance by blocking the genetic code or the membrane proteins responsible for drug-, or multidrug resistance.

The elimination of the genetic code of the resistance or inhibition of membrane transporter proteins were supposed to result in synergy with chemotherapeutics. Extrachromosomal genetic elements of prokaryotes and eukaryotes could be eliminated from bacteria and yeast and in addition expression of multidrug resistance gene was reduced in cancer cells in the presence of some heterocyclic compounds. Apparently the effective compounds could affect plasmid DNA and membrane function, as well. As a consequence some representative compounds such as calmodulin antagonists,

calcium channel blockers, proton pump inhibitors, etc. were able to synergize the effects of antibiotics. The resistance reversing effect was dependent on the chemical structure of the resistance modifiers. Based on the synergy between chemotherapeutics and non-conventional antibiotics we suppose that some resistance modifiers may help to overcome antibiotic resistance of bacteria *in vivo*.

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**Serological findings of children of HBsAg carrier mothers following
the introduction of preventive treatment upon birth**

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The complex hepatitis prevention programme has been introduced on the 1st of January 1995 in Hungary. Since that time all pregnant women are screened for the presence of HBsAg. Those found to be carriers are registered, educated and the newborn babies are immunized with hyperimmune gamma globulin and with recombinant HBsAg vaccine on separate injection sites.

Altogether 420.000 pregnant women were tested for the presence of HBsAg between 1995 and 1998 (about four years) in the 20 regional laboratories of the SERVICE. The number of HBsAg-carrier persons was 2135. The mean prevalence was calculated to be 0.5 per cent. There were observed, however, significant regional differences.

Up to the date of the last reporting 1.655 children became 18 months of age when the results of the active-passive preventive treatment can be serologically tested. The number of HBsAg negative children was 1642 (99.2%). The concentration of anti-HBsAg antibodies was found to be more than 10 miliunits (International Unit) in the case of 1.295 children (78%). Only 347 children had to be revaccinated.

Three of the 13 children (unsuccessful preventive treatment i.e. 0.8% of the children) were born from one single mother, who seroconverted last year, and the preventive treatment was also successful in the case of her 4th baby born in 1999.

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RfaH influences expression of the hemin receptor ChuA and *in vivo* virulence of a uropathogenic *E. coli* strain

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RfaH is a global regulator in *E. coli*, influencing expression of long operons encoding factors that are important for virulence and fertility. The affected operons contain a conserved motif termed JUMPStart sequence, the presence of which is required for the RfaH-derived regulation.

We report that RfaH – beside several other virulence determinants – affects expression of the hemin receptor ChuA in the uropathogenic *E. coli* strain 536. The *chuA*-transcript level was significantly reduced in an *rfaH* mutant compared to the wild type. The decrease of specific mRNA resulted in a lower amount of ChuA detected in Western blot. In the *rfaH* mutant strain, the decrease in the amount of hemin receptor, however, did not significantly alter the ability to utilize hemin as an iron source in comparison to the wild type strain. Nevertheless, *in vivo* virulence in a mouse model as well as serum resistance of the *rfaH* mutant were significantly lower than those of the wild type strain. Complementation of the mutant 536*rfaH* with a plasmid carrying an intact *rfaH* gene, restored higher *chuA* transcript- and ChuA receptor levels as well as increased virulence and serum resistance.

The nucleotide sequence of *chuA* along with its 700 bp 5'-flanking region was determined. A motif resembling the JUMPStart sequence was identified within *chuA*. Furthermore, we identified two variants of the *chuA* determinant differing in their neighbouring regions and exhibiting a well-defined distributional pattern among different pathogroups of *E. coli*.

Since RfaH was reported to be a major regulator of α -haemolysin, we propose a common regulatory pathway of the toxin that gains access to the intracellular heme reservoir and the hemin uptake system. This would be energetically advantageous for pathogenic bacteria since utilization of heme compounds liberated from eukaryotic cells is considered to be an important iron acquisition strategy during infection.

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Sequence analysis of porcine adenovirus serotype five

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The complete nucleotide sequence of porcine adenovirus serotype-5 (PAdV-5) was determined and the transcription map was constructed. The size of the genome was found to be 32 621 nucleotides. The early promoter regions, the major late promoter (MLP) and polyadenylation sites were predicted and the early and late transcription units were determined. The PAdV-5 genome could be divided into four early and six late transcription units. The E1A 173R (19.88 kDa) and E1A 67R (7.76 kDa) proteins were encoded by two overlapping open reading frames (ORF), which were similar in length to the CAdV-1 E1A proteins. In the E2B region, the full length of the precursor terminal protein (pTP) and the DNA polymerase genes were determined by sequence alignment to known adenovirus pTP and DNA polymerase cDNA sequences.

The MLP of PAdV-5 was similar to the MLPs of human adenoviruses and other previously studied mastadenoviruses. The canonical TATA-box of the MLP was located at nt 5122-5128, an inverted CAAT box (nt 5084-5088), an upstream promoter element (nt 5104-5109), initiator element (nt 5150-5156), and two downstream activating elements DE1 (nt 5225-5235) and DE2 (nt 5240-5255) were identified.

Twenty-seven putative proteins were located by their homology with other adenoviruses. The pVIII (79%), penton (77%) hexon (77%), and the protease (75%) proteins showed the highest identities to known adenovirus protein sequences. Further 8 genes were predicted according to their homology with hypothetical adenovirus genes and other viral and eukaryotic genes. Several special protein sequence motifs were characterized by their homology to similar protein motifs. The penton base protein lacked the integrin binding motif RGD, but the LDV motif was completely present. The fiber protein contained nineteen recognizable pseudorepeats. Interestingly, the highly conservative TLWT motif was missing from the predicted fiber protein. Based on sequence analysis and RNA secondary structure prediction, sequences for virus associated RNAs could not be recognized between the genes coding for pTP and 52/55kDa proteins.

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**Monitoring of influenza-like illnesses, and detection of influenza viruses
in Hungary, by using multiplex nested RT-PCR, in seasons 1998–99
and 1999–2000**

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About 300.000 patients with acute respiratory diseases consult their general practitioners in an average wintertime in Hungary. The illnesses occurred in different age groups in the two examined seasons: in 1998–99, and 1999–2000. The results of tests for respiratory tract infections in these two periods showed a conspicuous difference: in 1998–99 there was a regular influenza outbreak, but in 1999–2000 the outbreak was polyetiologic, different pathogenic agents were detected, mostly RSV, adeno-, parainfluenza viruses, and chlamydia and mycoplasma were also detected. Even the number of double-infections were higher in 1999–2000 than in the previous season.

The clinical samples (nasal-, or throat swabs in Virus Transport Medium, paired sera) sent to the Epidemiological Center's laboratory, were tested by numerous methods to detect the presence of respiratory tract viruses or confirm recent or past viral infections.

For isolation of influenza viruses we used the inoculation of samples in embryonated hens' eggs. Parallel to this, the multiplex RT-PCR was applied for detecting the viral RNA of influenza A and B in infected cells. The isolated influenza strains were similar to A/H3N2 Sydney 5/97, and the B/Beijing 184/93 prototype strains in 1998–99, and we have not isolated any influenza strains in 1999–2000. The advantage of the PCR method is that it provides a rapid typing and subtyping of influenza viruses in a single step. The multiplex RT-PCR was more sensitive than the inoculation in eggs: the percentage of positive results were ~10% with inoculation, and ~24% with PCR. 131 (in 1998–99) and 231 (in 1999–2000) samples were tested by direct immunofluorescence for adeno-, corona-, parainfluenza-, RS, Influenza A and B viruses, and for mycoplasma and chlamydia. Serological method was used in 113 (in 1998–99) and 232 (in 1999–2000) paired sera, for confirming recent or previous infections caused by adeno-, parainfluenza-, and RS viruses.

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Growth inhibition studies on *Salmonella typhimurium* under strict anaerobic conditions

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In the *Salmonella*-control programs of poultry, in addition to the commonly used prevention methods – last but not least on the basis of the successful application of Zoosaloral-H® – vaccination with live, attenuated *Salmonella* vaccines is becoming more attractive and applicable. A potential aim of vaccine development can be a *Salmonella* vaccine strain, which is able to induce cross-protection among the different *Salmonella* serotypes. An important finding of the last years: the colonization-inhibition can serve as a basis of the aim mentioned above. Colonization-inhibition means that if the gut of a day-old chick is colonized by a non-virulent, well-colonizing *Salmonella* strain, then this protects the chick against the infection with a second *Salmonella*. A parallel phenomenon with the colonization-inhibition is the growth-inhibition, found in the nutrient broth cultures of *Salmonella* strains. This means that inoculating a small amount of a given *Salmonella* strain into a stationary-phase nutrient broth culture of an other *Salmonella* strain, it is not able to grow and not because of the absence of available nutrients. For the deeper understanding of these two phenomena we have to identify the genes (and operons) that take part in these inhibitory mechanisms, for example by creating numerous mutants by transposon mutagenesis and investigating the Tn-insertion sites by PCR and sequencing them in the mutants that have lost their inhibitory capacity.

In poultry *S. typhimurium* F98 is a well-colonizing and strongly inhibitory strain. The authors have tested 2.800 *S. typhimurium* F98 Tn5-TcI mutants under strict anaerobiosis by adapting the standard aerobe growth-inhibition micro-plate assay to anaerobic conditions. The stationary-phase old LB-cultures of the mutants (marked by naladixic acid antibiotic resistance and stored on micro-plates) were inoculated by a special replica system with 10^3 – 10^4 cfu/ml amount of the spectinomycin resistance marked but isogenic *S. typhimurium* F98 parent strain. After 24 hours incubation the growth of the *S. typhimurium* F98 Spe^r was tested on bromothimol-blue plates containing Spe. Mutants that have lost their inhibitory phenomenon were selected. In this way we have found 34 non-inhibitory mutants and the characterization of these mutants and the comparison of our results with those that were found under aerobiosis is in progress. So far 26 mutants seem to have lost their growth inhibiting capacity under anaerobic conditions only.

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Pathogenicity and molecular characterization of *Ssp1* positive very virulent infectious bursal disease virus

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Infectious bursal disease virus (IBDV) is the etiological agent of infectious bursal disease (IBD) which causes significant losses to the poultry industries either by causing high mortality in an acute disease or as a consequence of immunosuppression in young chickens. On the basis of restriction fragment length polymorphism (RFLP) patterns, serotype 1 IBDV can be divided into 6 groups. Groups 1 and 2 consist of variant viruses, groups 3 and 4 consist of classical viruses, group 5 consists of the Lukert strain viruses and recently group 6 which consists of *Ssp1* positive very virulent IBDV (vvIBDV) from outside the Central and South America. Recently, we studied the molecular pathogenicity of three Malaysian, 92/04, 94/273 and 97/61 and one Bangladeshi 99/B551 isolates that are *Ssp1* positive. Pathogenicity studies of specific-pathogen-free (SPF) chickens infected with the respective isolates except 94/273 demonstrated typical clinical signs of vvIBDV including haemorrhage of the musculature and bursas with a mortality rate of 80% within 3 to 4 days postinfection. Fifty percent of the chickens also demonstrated moderate haemorrhage at the junction of the proventriculus gizzard region. On the other hand, SPF-chickens infected with 94/273 did not show any abnormal signs and no mortality was observed. Regardless the type of the isolates, all infected chickens show comparable histopathology lesion in the bursa and significant reduction ($p < 0.05$) of the average bursal weight; average body weight index at day 4 and 8 postinfection. Sequencing of the IBDV genome primarily the whole VP2 region (~1.35 kb) revealed that all the isolates have a conserve motif of SWSASGS at the hypervariable region. In addition, all the isolates have 3 aa residues, 222A, 256I and 294I that are unique to all vvIBDV. Another common feature for the isolates except 94/273 is the presence of *Styl* site at position 254, which encodes for amino acid (aa) glycine. This residue has been demonstrated among the vvIBDV, classical and vaccine strains. However, in the 94/273, the aa 254 undergo substitution from glycine to serine. Interesting, this aa variation has also been demonstrated in variant strains. The 94/273 also demonstrated an unique aa variation at position 270 from alanine to glutamic acid as found on serotype 2, OH strain. In addition, the aa at position 270 is also different among the STC and vaccine strains, where the alanine residue is substituted by threonine. Even though the 94/273 do not cause mortality in SPF-chickens, phylogenetic analysis of the VP2 gene classified the virus as vvIBDV with very close divergence to other vvIBDV from Malaysia as well as from Israel,

Hong Kong, Japan and China but differ from the Holland, Turkey and classical virulent USA isolates. In addition, based on the gross and histopathological data as well as other conserved RFLP markers it is unlikely that the 94/273 is a variant or attenuated strain. Even though the isolate has the three characteristic aa at the position 222, 256 and 294 as found on other vvIBDV, the aa variation at position 254 and 270 might be associated with the low virulence of the virus. So far, the well documented aa variations found in IBDV of low virulence is the reduction or lack of S residue at the conserve motif SWSASGS as well as the presence of aa residue 279N and 284T. However, both characteristic changes were not found in the 94/273. Currently, we are sequencing the whole genome of 94/273 for identifying further nucleotide changes. The discrepancy between molecular and pathological classification of IBDV strain 94/273 might be due to the constant evolution of the virus. Similar to other RNA viruses, it is likely that emergence of new IBDV with unique characteristic changes will be identified in the future.

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Study of fowl adenoviruses by PCR and DNA sequencing

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Until recently, all adenoviruses isolated from birds were classified into one genus called *Aviadenovirus*. This genus contained three groups (I–III) separating the viruses based on their different biological and immunological properties. By now, the genome of at least one representative from all the three groups has been fully sequenced. The comparison of these sequences revealed that the members of group II and III share more homology with certain adenoviruses of non-avian host origin, and should therefore be placed into other genera (*Atadenovirus*, 4th genus).

The genus *Aviadenovirus* (former group I) contains twelve chicken serotypes (FAdV 1–12), but DNA sequences were published only from four of these serotypes. A dependable phylogenetic analysis requires the usage of utmost homologous gene parts, therefore we aimed to obtain DNA sequences from all serotypes. We also wanted to estimate the variance within the genus for the correct definition of the new taxonomic category of the aviadenovirus species.

Raue and Hess (1998) reported a PCR method feasible for the amplification of two segments from the hexon gene from all the twelve FAdV serotypes. We have tested the primers on the prototype strains and four field isolates. The PCR products were sequenced, the received data compared and analysed by the PHYLIP programme package. The results will be presented by phylograms.

So far, we obtained DNA sequences from all but one isolate, from either both or one segment of the hexon gene. The level of variance within one serotype was found surprisingly high in some cases. For example, two extremely pathogenic strains (both of them isolated in Pakistan) from hydropericardium syndrome proved to be slightly different from each other and from the prototype FAdV-4 strain. Such kind of variance within one type has not been reported regarding viruses of other genera (e.g. EDS virus and HEV). We could not get amplification with serotype FAdV-5 as yet, although we have optimized the PCR conditions and prepared new virus stocks. It seems to be a failure of the primers, which had been originally designed considering only two sequences (FAdV-1 and 10) known at that time. This finding would suggest significant divergence of FAdV-5 (the single representative of species Fowl adenovirus B) from the other serotypes. We managed to amplify and sequence the viral DNA directly from a Hungarian field sample from the liver of a chick showing pathognomic lesions of inclusion body hepatitis. The amplified virus seemed to belong to species Fowl adenovirus E, but the serotype has not been defined yet.

Our results correlate well with the findings of Zsák and Kisary (1984), who had classified FAdVs into 5 groups based on restriction enzyme analysis. Their grouping became the basis of demarcation of the species (FAdV-A—E) of aviadenoviruses.

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Study on the suppression of *Listeria monocytogenes* by *Lactobacillus casei* in milk in function of the initial cellcount ratio and the incubation temperature

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There is increasing interest in the food industry for new biological preservation methods. The preservative effect of the different metabolites of lactic acid bacteria has been known for a long time. Numerous researchers studied the preventive effect of the

LAB metabolites to increase the safety of foods by suppressing the growth of food-borne pathogenic bacteria.

The aim of our work was to examine the possibility of growth of *Listeria monocytogenes* in milk in the presence of lactic acid bacteria. The pathogen is known to cause food-borne illness in raw or post-contaminated milk products (e.g. soft cheese).

In our study we examined the effect of the lactic acid production of *Lactobacillus casei* on the growth characteristics of *Listeria monocytogenes* in milk. Since the metabolic activity, thus the inhibitory effect of lactic acid bacteria is influenced by environmental factors, the interaction of the two microorganisms in the co-culture was examined at three different temperatures (7, 13, 20 °C). The initial cell count of *Lb. casei* varied from 10^3 cfu/ml, to 10^6 cfu/ml to result 1:1, 10:1, and 1000:1 ratio comparing to the number of *L. monocytogenes* (10^3 cfu/ml).

Based on the obtained growth curves a model was constructed on the growth characteristics of *L. monocytogenes* in the function of *Lb. casei* cell count and temperature.

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Characterization of *Neisseria meningitidis* isolated from Hungary by RAPD method

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Invasive diseases caused by *Neisseria meningitidis* occurred mainly sporadically in Hungary in the recent years. In December 1999 a community outbreak was observed in a military barrack, which concerned soldiers and civil population, too. To help in the investigation of epidemiological connections and to characterize the Hungarian isolates the Random Amplified Polymorphic DNA method was applied. Out of the 29 strains examined 22 belonged to serogroup C, 6 to B and 1 to W 13 5. Out of *N. meningitidis* strains of serogroup C 14 were isolated from the present outbreak and 4 during the previous months, 11 strains (4 C, 6 B, 1 W 13 5) were obtained before 1999. The RAPD examinations were performed using the modified method of Woods et al. [1]. As primers 1283 and 1290 were used, as templates the supernatants of boiled bacterial suspensions (1) or genomic DNA-s (2) prepared with Wizard kit were applied. The

amplicons were stained after agarose gel electrophoresis with ethidium-bromide. Determination of the sizes of amplicons was carried out in Gel Doc apparatus with Quantity One evaluation software using pGEM standard. It was found that the strains belonging to different serogroups could be differentiated with both primers on the basis of the size and number of their amplicons. While the differences within the strains of serogroup B and C isolated from sporadic cases before 1999 could be demonstrated with both primers, in case of the epidemic strains of serogroup C identical pattern was obtained with primer 1283, therefore these strains were studied with primer 1290, using templates type (1) and (2). Many amplicons appeared in both cases, but only a part of them was reproducible in repeated experiments, in accordance with literary data. For evaluation only the reproducible products were taken into account. The strains, isolated from the throat samples of 12 patients and 1 healthy person in December 1999 and January 2000 had identical patterns using template (1). The pattern of one strain differed, this was supported also by its different serotype. Dissimilar pattern was found in 3 strains of serogroup C, one strain was isolated in April 1999 and two earlier than 1999. Using template (2) 7 outbreak strains and 4 strains obtained before the outbreak belonged to identical group, but seven outbreak strains differed from this group in the number and size of their amplicons. Summarizing our results, it was found that the applied method was suitable to characterize the isolates and to follow the connections between the strains obtained during a given period.

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First detection of human calicivirus in a food-borne outbreak in Hungary

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Human caliciviruses (HuCVs) classified as members of the family *Caliciviridae* are a major cause of acute non-bacterial gastroenteritis cases and outbreaks in persons of all ages worldwide. They are subdivided into two genera: Norwalk-like viruses and

Sapporo-like viruses. The aim of the study was to detect HuCV infection in Hungary. We used reverse transcription-polymerase chain reaction (RT-PCR) with a primer pair p289/p290 designed upon the available sequence data of the conservative RNA-dependent RNA polymerase (ORF1) region of different HuCV strains from both genera. The definite identification was achieved by amplicon sequencing and phylogenetic analysis.

HuCV isolated from fecal samples collected from a food-borne outbreak in nurseries and elementary school in Szeged and Algyő areas in November 1998 caused a total of 80 illnesses with typical CV-related clinical symptoms and epidemiology. Phylogenetically, the HuCV/Szeged-Algyő/98/HUN strain belonged to the genogroup GII of Norwalk-like viruses and had 91% nucleotide sequence and 96% amino acid identity with the closest reference strain, the Lordsdale virus.

According to our knowledge this is the first detection of HuCV in a food-related epidemic in Hungary and Central-Eastern Europe, as well.

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Cationic lipid (DDA-Cl) + bacterial BRM complexes: increased survival times in tumorous mice

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Background: (i) The manipulative amplification of antitoxic immune response by DDA-chloride (a quaternary ammonium base: cationic lipid) was applied first by one of us, in 1968 [1]. Nowadays cationic lipids are widely used as vectors for gene therapy [2]. (ii) The role of BRMs of bacterial origin was also investigated previously by us in experiments aimed at increasing the level of both the antitoxic-type immunity and the non-specific resistance of tumor-infested mice [3–5].

At present the antitumor activity of bacterial BRM-s (biological response modifiers of bacterial origin: bacterium vaccines and derivatives) complexed with the cationic lipid DDA-chloride was defined. Mice (18 g each) were infested with 10^8 living cells of EHRLICH ascites tumor. Therapy with the above-mentioned complexes was done on -3 and + 3 days with the time of tumor-infestation on 0 day. Medium Survival Time (MST, in days) was defined in each group.

Biometrics: Kolmogorov and Smirnov two-sample test, ANOVA χ^2 "p" value of reliability. Detailed results are displayed in Table.

	N	MST	SD
Untreated controls	15	19.6	4.36
Controls treated with DDA-chloride	15	22.7	5.21
<i>C. parvum</i>	15	32.40	8.62
<i>C. parvum</i> + DDA-chloride	15	41.10	12.24
<i>C. granulosum</i>	14	35.60	11.96
<i>C. granulosum</i> + DDA-chloride	15	45.94	12.94
P40 (derivate of <i>C. granulosum</i>)*	14	32.73	6.51
P40 + DDA-chloride	15	44.40	10.93
<i>Brucella abortus</i>	15	28.06	6.41
<i>Brucella abortus</i> + DDA-chloride	15	38.66	8.44

* Courtesy of Institute Pasteur, Paris

Results: MST values of EHRLICH tumor infested mice increased significantly following bacterial BRM-treatments. An additional significant increase was observed following BRM-DDA-chloride treatment. DDA-chloride alone did not affect survival. *C. granulosum* + DDA-chloride complex exhibited the most pronounced life-span prolonging activity. Biometrics proved a high and statistically significant degree of homogeneity and reliability of results in all cases.

Summary: According to our observations, the useful gene therapy vectors, cationic lipids significantly improved the effectivity of bacterial BRM-based antitumor therapy in tumorous animals.

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Increasing the oncolytic activity of *IBDV* virus by traditional and cationic liposome complexes

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Introduction: Authors recently reported the beneficial effect of liposomes on the oncolytic activity of certain viruses [1, 2]. At present they compared the supposed oncolysis-increasing activity of Infectious Bursal Disease Virus (strain: IBDV/ BLB-90; -single dose: 5×10^5 TCID₅₀), complexed with "traditional" liposomes containing lecithin, cholesterol and stearylamine [3] and with a cationic lipid, DDA-chloride, which was used first as an immuno-modulant agent [4]. Nowadays cationic lipids are widely used as vectors for gene therapy [5].

Methods: Swiss albino mice infested with 10 g living cells of EHRlich ascites tumour were treated with "plain" IBDV and IBDV complexed with DDA-chloride and/or traditional liposome. Values of Medium Survival Times (MST, in days) were analysed statistically to define Student's $p\%$, ANOVA χ^2 , reliability $p\%$. Results are disclosed in Table.

Treatments	N	MST	SD
Untreated controls	16	19.6	4.3
IBDV	15	40.8	11.1
IBDV + Liposome	16	60.1	22.1
IBDV + DDA-chloride	16	55.0	15.1
IBDV + (Liposome + DDA-chloride)	15	59.2	13.5

Results unequivocally prove that the MST values increased significantly following treatment of tumour-infested mice with "plain" IBDV. A more pronounced increase of MSTs were achieved following application of DDA-chloride and/or traditional liposomes. ANOVA χ^2 and the reliability $p(\%)$ proved the homogeneity and reproducibility of the experimental results.

In summary, the oncolytic activity of IBDV was significantly enhanced in a complex with liposomes. Although in gene therapy cationic lipids are preferred as vectors in order to neutralise the negatively charged DNA, during our experiments we could not detect significant difference between the effects of the investigated liposome

complexes: DDA-chloride-Liposome-complex exhibited the same oncolysis-enhancing/immuno-modulant activity as the liposome or *DDA-chloride* alone.

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M. RUPNIK

Variability of *Clostridium difficile* strains

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Clostridium difficile is an anaerobic, sporogenic Gram-positive bacterium. It causes intestinal infections as antibiotic associated diarrhoea (AAD) and pseudomembranous colitis (PMC). The main virulence factors are two large toxins, toxin A (TcdA, enterotoxin) and toxin B (TcdB, cytotoxin). In our studies we have established a method for differentiation of *C. difficile* strains into groups called toxinotypes based on differences in their toxin genes. Toxinotypes have proven to be a good basis for studying pathogenesis, virulence factors and molecular biology of *C. difficile*.

Region coding for both toxins in *C. difficile* is called pathogenicity locus (PaLoc) and consists of five genes, two of them are toxin genes (tcdA and tcdB) and three additional genes are associated with regulation and transport (tcdC, D and E). The entire 19 kb sequence of PaLoc is known for reference strain VPI 10463. We use ten overlapping fragments for amplification of PaLoc region. Two of them amplifying 5'-region of tcdB gene and 3'-region of tcdA gene, are used routinely in screening for variant strains. The changes observed in PaLoc region are deletions, insertions and polymorphism of restriction sites. Toxinotype is a group of strains with identical changes in PaLoc when compared to reference strain. At the time fifteen toxinotype's are known (I to XV).

Studies of variant *C. difficile* strains have contributed significant information in different research areas. For instance, a group of variant strains produce only TcdB. This variant toxin B is changed and resembles in its properties to *C. difficile* toxin as well as *C. sordellii* toxin. As such strains are reported also to cause disease, they have

changed the dogma that both toxins act synergistically and are needed for full virulence. Therefore the diagnostic tests of *C. difficile* are currently changing. Further, a new form of genetic element was found in variant strains of *C. difficile* (toxintype XIV) combining the properties of intron and insertion element. And finally, only strains with changed toxin genes were shown to produce a third toxin, binary toxin.

E. RUSVAI, J. BROJNÁS, I. MEZEY, J. DANKA, GY. BERENCSI

A high percentage of the Hungarian young population is not protected against HAV

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A seroepidemiological study was conducted in Hungary in 1999–2000 to determine the seroprevalence of anti-HAV in the general population.

5216 subjects (2528 men and 2688 women) were stratified by age 0–5, 6–19, 20–29, 30–39, 40–49, 50–59, more than 60. The presence of total anti-HAV antibody was investigated using commercial enzyme immunoassay (Hepanostika HAV antibody Organon Teknika).

22.57% of the subjects were found positive for anti-HAV antibody. Prevalence of seropositivity increased with increasing age, being 2.19%, 4.44%, 8.35%, 22.79%, 54.31%, 81.94%, 91.50% for the various age groups, respectively.

The results show a large number of susceptible subjects in the young adolescent population, which could cause public health problem in the country during disasters.

K. SALÁNKI, E. HUPPERT, E. BALÁZS

The compatibility of the 3a protein and the coat protein of cucumoviruses is required for cell-to-cell movement

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It is well known that movement protein and coat protein are both essential for cell-to-cell movement of cucumoviruses, but the exact function and the possible interactions of the proteins are not characterized yet.

In our previous work we constructed recombinant viruses between two cucumoviruses, the cucumber mosaic virus (CMV) and tomato aspermy virus (TAV) by exchanging the 3A protein and the coat protein of the two viruses. The recombinant

containing the 3A protein of CMV and the coat protein of TAV caused systemic infection on different test plants, while the other recombinant containing the 3A protein of TAV and the coat protein of CMV was not able to move from cell-to-cell, however it was able to replicate in protoplast system.

In our present work we constructed further recombinants between the two viruses in order to identify which parts of the two proteins should be compatible for the local movement of the viruses. In case of the 3a protein we chose the recombination sites based on the sequence similarity of the two proteins, while in case of the coat protein we selected the sites for recombination by the known three dimensional structure. The recombinant virus unable to move from cell-to-cell was able for local movement if we exchanged the carboxy terminal 30 amino acids of the TAV 3A protein for the similar region of the CMV 3A protein, or if we replaced the amino terminal 70 amino acids of the CMV coat protein for the similar part of the TAV coat protein. So the compatibility of the carboxy terminal part of the 3A protein and the amino terminal part of the coat protein is required for the cell-to-cell movement of cucumoviruses, and presumable the interaction of these two proteins is necessary in this process.

J. SZABÓ¹, P. EBBESEN², A. BÁCSI¹, Z. BECK¹, I. ANDIRKÓ¹, F. D. TÓTH¹

Infection of human syncytiotrophoblast with pseudotypes of vesicular stomatitis virus bearing envelope antigens of various HIV-1 strains

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Mother-to-child transmission is clearly an important mode of spread for human immunodeficiency virus type 1 (HIV-1). The placental syncytiotrophoblast (ST) forms a continuous multinucleated epithelium in direct contact with maternal blood. Although HIV-1 has been detected in the ST layer *in situ*, there is conflicting evidence regarding infection of ST cells with cell-free virus. We used the phenotypic mixing between HIV-1 and vesicular stomatitis virus (VSV) to assay the susceptibility of human ST cells to penetration by various HIV-1 strains. We found that VSV(HIV-1_{IIIB}) and VSV(HIV-1_{Ba-L}) pseudotypes entered ST cells, whereas VSV pseudotyped with envelope antigens of RF, MN or Ada-M strains of HIV-1 did not infect ST cells. Neutralizing sera to HIV-1_{IIIB} and HIV-1_{Ba-L} were able to completely inhibit entry of VSV(HIV-1_{IIIB}) and VSV(HIV-1_{Ba-L}) into ST cells. These findings indicated that

infection of ST cells with VSV(HIV-1) pseudotypes was mediated by Envs from IIIB and Ba-L strains of HIV-1. Monoclonal antibodies (MAb) to CD4, CXCR4, CCR5 and CCR3 were tested for their ability to block VSV(HIV-1) infection of ST cells. Neither the anti-CD4 nor the anti-CXCR4, anti-CCR5 and anti-CCR3 MAbs had any inhibitory effect upon infection of ST cells with VSV(HIV-1) pseudotypes. Our data suggest that ST cells can be infected with cell-free HIV-1 and the susceptibility of ST cells to penetration by the virus is strain dependent. The mechanism of entry into ST cells remains to be revealed.

D. SZAKÁL, K. SZÖKE, T. PÁL

The colony immunoblot method adapted for detecting *Shigella* and EIEC from milk

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Dysentery-causing *Shigella* and EIEC strains usually spread by the feco-oral route, but frequently contaminated water and food, particularly milk and dairy products are the vehicles of transmission. The identification of these bacterial agents from these samples is rather laborious by the classical laboratory methods. A rapid Ipa-C specific colony immunoblot method has already been successfully used for the detection of these bacteria from water and stool samples. This work presents its successful adaptation for milk samples.

Samples contaminated artificially with *Shigella* or EIEC were tested by the colony immunoblot technique, with the necessary changes of the method due to the consistency of the samples. As a control technique, samples were spread onto selective media, followed by IpaC-specific ELISA of selected colonies. Our experiments showed that the colony blot method was equally sensitive as the other method, but was significantly faster.



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Leptospirosis: state of epidemiology, diagnostical news

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Leptospirosis has been considered to be a relatively infrequent disease in Hungary up to now. The number of diseases caused by *Leptospira interrogans* has been increased presumably due to the rainier weather of late years and the multiplication of rodents, mainly of rats. In 1997 out of 2141 examined samples 91 (4%), in 1998 out of 1586 61 (4%), whilst in 1999 out of 2331 341 samples (15%) have been proved to be positive by the test of lysis-agglutination. The examination by lysis-agglutination is applied routinely only in the OEK and in the Institute of National Public Health of Hajdú-Bihar county, Debrecen, because of the increased danger of infection, and as special culture-medium and evaluation by microscope of dark visual field is needed.

In the last two years a comparative examination was carried out among the following commercial tests: two ELISA, one IF and one antibody revealing LATEX agglutination test. The examined ELISA kits did not fulfil the expectations. The results received by the IF and LATEX tests showed outstanding agreement with those obtained by lysis-agglutination. On the basis of the examinations the IF and LATEX tests seem to be suitable for screening tests in the laboratory diagnostic of leptospirosis but for confirmation and serotyping the lysis-agglutination method of the two above-mentioned laboratories should be used in the future, too.

GY. SZLADEK, J. KÓNYA, A. JUHÁSZ, K. SZARKA, L. GERGELY

The prevalence of TT virus (TTV) in renal transplant recipients

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TTV is a novel unenveloped DNA virus first isolated from a Japanese patient with fulminant liver disease of unknown origin. The virus has a single stranded circular DNA genome, and so is thought to belong to the Circoviridae family, and related with chicken anaemia virus (CAV), porcine circovirus (PCV), beak and feather disease virus (BFDV). The pathogenic role of the human circovirus TTV is yet poorly known. TTV

can be detected also in the peripheral blood of 5–20% of the healthy blood donors. TTV has recently been reported to be excreted via faeces, although data indicating faecal-oral transmission are not available.

To detect the viral DNA in DNA extracted from peripheral blood mononuclear cells (PBMC) of renal transplant recipients and healthy blood donors we used the polymerase chain reaction (PCR) method with primers designed for the ORF I region of the viral genome. Our results show that the prevalence of TTV is significantly higher in renal transplant recipients [61% (53/87)] than in healthy blood donors [19% (5/26)]. The cause of the higher prevalence detected in renal transplant recipients may be attributed to the blood products received because of the illness, to the transplant organ, or the immune suppression.

K. SZŐKE, J. KÓNYA, K. SZARKA, GY. VERESS, A. JUHÁSZ, L. GERGELY

Quantification of human cytomegalovirus DNA using the polymerase chain reaction

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In organ transplant recipients the latent cytomegalovirus (CMV) infection can be easily reactivated because of the immunosuppression. The most important diagnostic sign of the reactivation is the developing viremia. The viral gene-expression or the detection of viral structure proteins by antigenemia test can indicate the replicating virus in the peripheral blood. The viral genome can be detected in low copy number in the latent phase of the infection because in this phase the viruses are mostly in the circulating monocytes. In the viremic phase the viral genome can be detected in the plasma or if the viral DNA is tested in whole blood then the amplification of the viral genome can be. Our goal was to develop a quantitative PCR method, which is based on the competition of a mutant sequence developed by us and the wild sequence. The oligonucleotide primers amplified a 149 bp long section from the glycoprotein B gene. The amplicon was cloned (wild sequence) thereafter a 200 bp long indifferent insert was ligated in to the *Bgl* II cleavage site inside the cloned sequence (mutant sequence). The amount of cytomegalovirus DNA was measured in the peripheral blood of organ transplant recipients. We detected elevated CMV DNA level in the blood of transplant patients during the antigenemia positive phase. In the non-antigenemia phase the DNA levels of the transplant patients were in the same range as those of the healthy blood donors.

In conclusion, we developed a quantitative PCR method, that is to be tested on clinical samples in large scale.

E. SZIGETI¹, J. FARKAS²

Use of a conductimetric technique for data capture in predictive microbiology

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Conductimetry as an alternative data capture method for following microbial growth has a great potential as a research tool of predictive microbiology. In spite of this fact there is only a limited number of applications using conductimetric data for model generation. In this study the growth of single strains of *Listeria monocytogenes* and *Lactococcus lactis* was tested in 5 media using a RABIT[®] instrument. The goal of the work was to find selective growth media for *Listeria* and *Lactococcus*, respectively, in order to study their interaction in mixed-culture using the conductimetric technique. Whitley Anaerobic broth, Whitley Impedance broth and modified Whitley Impedance broth (Whitley Impedance broth + Chloramphenicol (7 mg/l)) were not suitable for following selectively the growth of *Lactococcus lactis* or *Listeria monocytogenes* in a mixed culture of the two bacteria. BiMedia 630 A for *Lactococcus lactis* and Bimedia 403 A for *Listeria monocytogenes* satisfied the demands raised by conductance measurement. Linear correlations were established between the graphically estimated detection time (TTD) values of the conductance curves and the logarithmic numbers of colony forming units (cfu). The correlations were very strong in each case (determination coefficients (R^2) of the linear regression were higher than 0.98 at both medium-strain combinations). However, in BiMedia 630 *Listeria monocytogenes* was capable of slow growth, therefore, this medium will be suitable for studying microbial interactions only when *Listeria* are present in the mixed culture in concentrations less than 10^6 cfu/ml.

E. SZIGETI

Interaction between *Lactococcus lactis* strains and *Listeria monocytogenes* in mixed-cultures

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Competition of a bacteriocinogenic and a non-bacteriocinogenic *Lactococcus lactis* strain and a *Listeria monocytogenes* strain was studied in two semi-synthetic liquid media at various temperatures. The media used for the study were ST I and modified ST I broth (ST I broth + 1 g/l Tween 80). Incubation temperatures were 30 °C, 20 °C and 10 °C. In this study the role of inoculum levels of the competing strains in the suppression of growth of *L. monocytogenes* was investigated. Furthermore, the bacteriocin activity of nisin produced by the bacteriocinogenic *Lac. lactis* strain against *L. monocytogenes* was determined in the mixed cultures of the two bacteria.

The competition studies showed that the growth of the bacteriocinogenic *Lac. lactis* and *L. monocytogenes* in a co-culture did not differ significantly in ST I and modified ST I broth at 30 °C, when the initial cell numbers of the two bacteria were about equal. In both media, the inactivation of *L. monocytogenes* could occur only when the cell concentration of the bacteriocinogenic competitor reached the level of at least 10^7 cfu/ml required to produce sufficient bacteriocin concentration. Although bacteriocin activity was not detectable against *L. monocytogenes* when the co-culture was inoculated into ST I broth, the decrease of the viable cell count of *Listeria* seemed to follow the same manner as in modified ST I broth. This observation suggests that the bacteriocinogenic *Lac. lactis* also produced nisin in ST I broth, although this activity was not detectable by the agar spot test.

When the two competitors were present in the same initial level (approx. 10^5 cfu/ml), the non-bacteriocinogenic *Lac. lactis* did not affect the growth of *L. monocytogenes* at 30 °C in modified ST I broth. I conclude from this observation that the considerable decline of the viable cell counts of *L. monocytogenes* found in the aforementioned case was not caused alone by the lactic acid produced by *Lac. lactis*.

From the competition studies with different initial cell numbers of the bacteriocinogenic strain and *L. monocytogenes* at 30 °C, 20 °C and 10 °C in the modified medium the following conclusions can be drawn: The two bacteria grew in a mixed culture the same way as in their monocultures at 30 °C, until nisin reached the detectable concentration, when a five log-cycles decrease of the viable count of *L. monocytogenes* occurred. The same phenomenon was also observed when the initial level of the lactic acid bacteria was one log-cycle higher or lower than that of the

Listeria. The effect of nisin production was noted only in the earlier or later induction of *Listeria* inactivation.

Incubation of the mixed cultures at 20 °C gave similar results but the bacteriocinogenic activity resulted only in a three log-cycles decline of the cell count of *L. monocytogenes*. Furthermore, the suppression of the pathogen was induced much later at this temperature than at 30 °C.

At 10 °C *Lactococcus lactis* produced much less bacteriocin than at 30 °C, therefore a drastic decrease of the listeria cell count was not observed. Suppression of the *Listeria* growth was expressed in its decreased maximum population level (i.e. in an earlier appearance of the stationary phase).

M. TAKÁCS¹, E. RUSVAI¹, J. BROJNÁS¹, G. TÓTH², A. SZENDRŐI¹, GY. BERENCSEI¹

Prevalence of TTV and HGV in Hungary

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The majority of the viral hepatitis is caused by five hepatitis viruses (A, B, C, D, E). In 1995 three new flaviviruses were discovered, GBV-A, GBV-B and GBV-C (also known as HGV) viruses. TT virus was discovered in 1997. To reveal the RNA of the GBV-A, -B, and HGV viruses in the sera of patients, RT-PCR was applied using the primers published in the literature. Seminested PCR was used to detect the DNA of TTV. The nucleotide sequences of nested PCR products were determined.

Specific PCR products were not found in cases of GBV-A and GBV-B viruses in accordance to the literature. The RNA of the HGV virus was found in the sera of patients suffering from hepatitis of unknown origin or from aplastic anaemia. Twenty-one sera of 252 were positive. Eight PCR products were sequenced and these sequences were found to be different from each other. In case of this virus, sequences of 2000 nucleotides are needed to construct a reliable phylogenetic tree. It could not be done, because the length of the PCR products was only 400 nucleotides. More tests and sequences are needed for further studies.

The antibodies of the GBV-C virus were found in the sera of patient suffering from hepatitis of unknown origin or aplastic anaemia. Seventy-four sera were tested and 21 were found to be positive. The sera of 255 healthy persons above 60 years were tested for the presence of GBV-C antibodies, and 71 were positive.

Eighty-five sera of patients with hepatitis of unknown origin were tested for the presence of TTV-DNA. Thirty of them were positive. Seven PCR products were

directly sequenced. Four sequences were homogeneous, 3 indicated mixed infections. A phylogenetic tree was constructed from sequence of the main genotypes and from our data. In each case the genotype 2 was found in our samples. Mixed sequences will be cloned.

I. TÓBIÁS¹, L. PALKOVICS², L. TZEKOVA², E. BALÁZS²

Replacement of coat protein gene of PPV infectious clone by zucchini yellow mosaic virus coat protein does not extend the virus host range

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Many informations are available on the consequences of gene transfer between distantly related viruses in different virus genera but not in the potyvirus genus. In this study two members of the potyvirus genus, plum pox virus (PPV-SK68) and zucchini yellow mosaic virus (ZYMV) were used. Both of these viruses have different host range, pathogenicity and the size of their CP is also different (PPV CP is 990 nt and ZYMV CP is 837 nt long). Comparison of the nucleotide and amino acid sequence of these two isolates showed 58% and 65% homology, respectively. PPV infects mainly *Prunus* species while ZYMV infects *Cucurbitaceae* plants. Both viruses have different herbaceous test plants, too. To investigate the function of the coat protein of potyviruses for pathogenicity and host specificity the coat protein gene of an infectious plum pox virus clone was replaced by the ZYMV CP gene. Three different constructs were analysed but only one was infectious when the secondary structure between the CP and the 3' NTR could be formed. This hybrid clone was infectious on *Nicotiana benthamiana* and *Nicotiana clelandii* plants and the hybrid nature of this virus was verified by Western blot using specific PPV and ZYMV antibodies.

Our results provide direct evidence that the coat protein is exchangeable between these two potyviruses, the coat proteins have similar functions and this heterologous ZYMV CP is able to cooperate with other proteins of the PPV.

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Virulence markers of human uropathogenic *Escherichia coli* in Hungary

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A collection of 190 human uropathogenic *Escherichia coli* (UPEC) and 12 other extraintestinal *E. coli* strains have been investigated for the presence of genes of cytotoxic necrotic factors (CNF) and cytolethal distending factors (CDT) using specific PCR primers. As a result, 42 (22.1%) of the UPEC strains were CNF1⁺, 12 strains were CNF1⁺ and CDT⁺, and 3 strains were CDT⁺ only.

CNF and CDT production of selected strains was proven by HeLa cell assay. When haemolytic abilities were tested as much as 80 strains (42.1%) proved to be haemolytic. Extraintestinal strains were negative for both CNF and CDT (3 of them were haemolytic). The presence of *pap*-, *sfa*- and *afa*-adhesin genes was determined by multiplex PCR which resulted in a 86.6% positivity for *sfa*, 71.1% positivity for *pap* and no strains were detected with *afa* genes. As many as 44 strains contained several virulence genes. These virulence genes have formed 7 virulence clusters, the most frequent being the CNF1⁺, P⁺, S⁺, Hy⁺, in which the third of the strains was CDT⁺ as well.

These results suggest that CDT is a virulence marker characteristic for human UPEC.

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Urovirulence markers of *Escherichia coli* isolated from cases of postweaning diarrhea and oedema disease of swine

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Postweaning diarrhea of swine is a multifactorial disease in which enterotoxigenic *E. coli* (ETEC) is playing a crucial role. Verotoxigenic *E. coli* (VTEC) in swine is known to produce oedema disease. Both porcine ETEC and VTEC have their characteristic

adhesive fimbriae (F4, F18). The goal of these studies was to see whether further toxins and adhesins could also play a role in postweaning diarrhea. Here we have tested 204 such *E. coli* strains by PCR for the presence of CNF genes and found 12 *cnf1*⁺ strains (5.9%) that were all haemolytic. The phenotypic expression of CNF1 was confirmed by HeLa cell assay as well as in rabbit skin test. The 12 CNF1⁺ strains were further PCR tested for the presence of genes of cytolethal distending toxin (CDT) and *pap* and *sfa* fimbria, and all were found to harbour genes of either or both fimbria, but none possessed *afa*. On the other hand, 10 haemolytic postweaning porcine intestinal isolates which were *cnf*⁻ were also negative for *pap* or *sfa* fimbrial genes. Further studies of 10 extraintestinal porcine *E. coli* strains detected 3 *cnf1*⁺ strains, one of which had both *pap* and *sfa*, while the others had either *pap* or *sfa* genes.

These results confirm two earlier reports on the presence and clustering of urovirulence characters among porcine intestinal and extraintestinal *E. coli* strains and indicate that CDT can also be a virulence factor of porcine enteric *E. coli*.

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Methicillin-resistant staphylococci in a County Hospital in Hungary

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Objectives: To assess the frequency and the distribution of methicillin-resistant *Staphylococcal* (MRS) infections in a 1521-bed county hospital.

Methods: Clinical, epidemiological and bacteriologic observations related to MRS infections were made.

Results: Between January 1999 and October 1999, 1323 different *Staphylococcus* strains [815 *Staphylococcus aureus* (SA) and 508 coagulase-negative *Staphylococcus* (CNS)] were isolated from clinical sources, out of which 207 (15.6%) specimen proved to be methicillin-resistant. Methicillin resistance is especially frequent among CNS strains (185 were methicillin resistant out of 508 – 36.4%) while that is far less among SA strains (22 out of 815 – 2.7%).

Out of the 174 cases investigated only ten patients were infected with MRS strains, and the vast majority of patients were only colonized without infections. The sites of infections were wounds (4 MRSA, 1 MRCNS), blood (1 MRSA, 1 MRCNS) respiratory tract (1 MRSA) urinary tract (1 MRSA), ventriculo-atrial shunt (1 MRCNS).

The sites colonization with MRS strains were blood (40 MRCNS), wounds (41 MRCNS, 5 MRSA), urinary tract (15 MRCNS, 1 MRSA) respiratory tract (58 MRCNS, 1 MRSA, others (3 MRCNS).

Resistance of the MRS strains to various antibiotics was studied with disk susceptibility tests. All strains were still susceptible to glycopeptides. 5.9% of the MRS strains were aminoglycoside-resistant and 20.3% fluoroquinolone-resistant.

Conclusions: Fortunately, infections due to MRS strains are not frequent in our hospital, but the prevalence of colonization with MRS strains are quite high and under certain circumstances these strains may become pathogens.

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Cloning and sequencing of the genome of the ovine adenovirus 1

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There are 7 adenovirus serotypes isolated from sheep. Five of them (OAdV-1 to 5) belong to the genus *Mastadenovirus*. The isolate OAV287 is proven to be the member of the genus *Atadenovirus* although this type has not received a serotype number yet. The taxonomic position of OAdV-6 is so far unclarified. OAdV serotypes 2–5 belong to the same species (*Ovine adenovirus A*) according to the newest DNA sequence comparisons and phylogenetic calculations. Based on the restriction enzyme digestion patterns and on the results of hybridization experiments with the DNA of OAdV-3, it is most likely that OAdV-1 is a specimen of another species. We have decided the detailed investigation of the genome of this serotype.

The virus DNA was cut with the restriction enzyme *Pst*I. The fragments were cloned randomly into plasmid vector. The isolated clones contained more than half of the virus genome inserted. The clones were sequenced in the Central Veterinary Institute. We obtained more than 8000 bp DNA sequence data so far by sequencing both ends of each fragment. The examinations carried out with these sequences confirmed our preliminary assumption that OAdV-1 belongs to a distinct species (*Ovine adenovirus B*) of the genus *Mastadenovirus*.

Support: Tét Balaton

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HHV-7 and human parvovirus B19 in "gloves-and-socks" syndrome

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Nearly 30 cases of papular-purpuric "gloves-and-socks" syndrome (PPGSS) in the Europe and the USA have been known since 1990. Its ethiology is unknown, although HPV B 19, morbilli, CMV, Coxsackie B6, HBV and HHV-6 infections were described. In adolescence and early adulthood after prodromes symmetric, painful papules and purpures develop on the swollen skin followed by healing without scars in one or two weeks.

High fever, leukopenia with relative lymphocytosis, microhematuria, prolonged Quick-time, elevated level of liver enzymes accompany the clinical picture. Authors here describe the case of a 13-year-old girl, and independently from her an other family with symptom-free mother, but disease in the 29-old father and their nearly two-year-old son. Up to date, they are the oldest and youngest patients described. Antibody levels of the girl were: HHV-6B IgM, IgG 1:20, HHV-7 IgM, IgG >1:640, HPV B19 IgM positive, EBV negative. In the serum sample taken five months later from the son of the family, HHV-7 IgM <1:10, high avidity (HA) IgG and total IgG 1:80, HPV B19 negative; in the father HHV-7 IgM 1:80, HA-IgG and IgG 1:160, HPV B 19 IgG positive; in the mother HHV-7 IgM < 1:10, HA-IgG and IgG 1:160, HPV B 19 negative were. HHV-7 PCR from their peripheral blood lymphocytes: strong positivity was found in the mother and son, but very faint in the father. Based on these results, one can presume that in case of the 13-year-old girl interactions of the simultaneous HHV-7 and HPV B 19 infections might contribute to the clinical outcome. In case of the son and father, primary HHV-7 infection accompanied by HPV B 19 and possibly another virus infection might induce symptoms. In such a rare disease, it would be desirable to study infection by a large panel of viruses to reveal the exact pathomechanism.

*Medical student. Supported by OTKA T-029299.

GY. VERESS, K. SZARKA, M. MURVAI, J. KÓNYA, A. JUHÁSZ, L. GERGELY

Transcriptional activity of human papillomavirus type 16 variants having deletions in the long-control region

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Transcription of the E6 and E7 viral oncogenes of human papillomavirus (HPV) type 16 is regulated by the P97 major early promoter and enhancer and silencer elements found in the long-control region (LCR). The central part of the LCR contains an epithelial-specific enhancer, while the 3' part contains two adjacent silencer elements: one which is YY1-dependent and another which is down regulated by the differentiation-specific factor CDP/Cut.

In this study, we wanted to check the transcriptional activity of natural HPV 16 variants having long deletions in the LCR. The HPV 16 LCR regions were amplified from HPV 16 positive invasive cervical cancer specimens and cloned into the promoter-free reporter vector pALuc. Transcriptional activity of the different clones was measured by luciferase test after transient transfection into HeLa cells.

From case #936, in addition to a full-length LCR clone (936LCR), two deletion variants were isolated. One variant (936LCRd1) contained a 212 bp deletion (nt 7521 – nt 7732) in the tissue-specific enhancer region. The other variant (936LCRd2) had a 378 bp deletion (nt 7564 – nt 35) encompassing a great part of the enhancer and both 3' silencer regions. In spite of these deletions, both variants had comparable transcriptional activity to that of the full-length 936LCR clone. From the other case (#33), again a full-length clone (33LCR) and two deletion variants were isolated. A large part of the enhancer region and the total YY1-dependent silencer region were deleted (292 bps, nt 7566 – nt 7857) in isolate 33LCRd1. This clone had a reduced activity compared to the full-length 33LCR clone. The other deletion variant (33LCRd2) contained a 563 bp deletion (nt 7363 – nt 19) encompassing the total enhancer and 3' silencer regions. In fact, this clone contains only the 3' part of the L1 gene and a 210 bp upstream LCR element, in addition to the P97 promoter region. Interestingly, 33LCRd2 had about fourfold higher transcriptional activity than the full-length 33LCR clone.

In conclusion, deletions of both the YY1-dependent and the CDP-dependent silencer elements of the HPV 16 LCR seem to be responsible for possible mechanisms to up-regulate the expression of E6 and E7 oncogenes in cervical cancer. In addition, our results also point to the existence of a novel transcriptional enhancer element at the 5' part of the LCR.

I. ZDOVC, M. OCEPEK

Presence of *Rhodococcus equi* in lymph nodes of pigs suspected on avian tuberculosis

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Objectives: Avian tuberculosis of pigs is quite common disease in Slovenia and it is very important from the viewpoint of food hygiene. It is a contagious disease and a potential zoonosis. *Rhodococcus equi* is a facultative, intracellular, Gram-positive coccobacillus that causes chronic suppurative bronchopneumonia and enteritis in 2–4 month old foals and abscesses in older foals. The bacterium can multiply in soil enriched with equine faeces and may be a commensal in intestine. There are, however, some reports of the isolation of *R. equi* from the lymph nodes of pigs with or without lesions resembling those of tuberculosis. According to recent data *R. equi* has been recognized as a human pathogen preferably in immunosuppressed individuals. The purpose of this study was to investigate the presence of *R. equi* in pigs' lymph nodes suspected to *Mycobacterium avium* infections.

Material and methods: 45 samples of mesenteric and mandibular lymph nodes of pigs from different households were observed. Microscopically the lymph nodes displayed granulomatous lymphadenitis with epitheloid cell reminiscent of tuberculosis. The material was treated with classical method with N-acetyl-L-cystein (NALC). Sediment was inoculated on the following media: egg media with pyruvate (IUT-P), egg media with glycerol (IUT-G), Stonebrink, Middlebrook 7H10 and fluid media MGIT. The mycobacteria were determined by the classical methods, as well as with ACCUPROBE for *M. avium* complex. *R. equi* suspected colonies were subcultured on agar supplemented with 5% of ovine blood. A presumptive diagnose of *R. equi* was established initially based on Ziehl-Nielsen stain. Biochemical characteristics were tested by commercial system Api Coryne (BioMerieux, France) according to the producers' instructions.

Results and discussion: *Rhodococcus equi* were isolated from 17 (37.8%) of specimens studied while mycobacteria were isolated from 19 (42.2%) specimens. Both species of microorganisms were isolated from 4 (8.9%) specimens.

From our investigation it is obvious that *Rhodococcus equi* is frequently present in lymph nodes of pigs suspected to avian tuberculosis. Since *R. equi* causes similar gross pathology in lymph nodes as mycobacteria, the veterinary inspectors in slaughterhouses in such cases have to take all measurements as in cases of tuberculosis.

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Virus abundance in northern Adriatic sea

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Viruses exert significant control on marine bacterial and phytoplankton communities with respect to both biological production and species composition, influencing the pathways of matter and energy transfer in the system. Viruses infecting specific bacterial isolates from Northern Adriatic Sea, as well as virus community *in situ* were studied by electron transmission microscopy, epifluorescence microscopy and virus growth assays. Bacterial direct counts were determined by SYBR Green epifluorescence, chlorophyll a was measured spectrophotometrically. The experimental sites were characterized by different physico-chemical parameters (i.e. temperature, salinity, nitrogen and phosphorus content). The density profiles of viruses were determined in surface coastal waters, estuarine waters and saltpan waters. Temporal and vertical distribution of viruses were measured in coastal waters. The results indicate that viruses were consistently the most abundant biological group of microorganism in the Northern Adriatic Sea. Viral abundance ranged between 10^8 and 10^{10} per litre and was dependent on seasonal and diurnal cycles. Virus densities in a shallow Northern Adriatic Sea were only moderately correlated with depth, chlorophyll, temperature, salinity, nitrogen and phosphorus. However, in all cases studied viruses strongly correlate with bacteria. Viruses to bacteria ratio varied between 5 and 22 in different environments. In addition, 28 fast growing bacteria isolated from different environments of Northern Adriatic Sea were examined for lysogeny by treatment with Mitomycin-C. After prophage induction 71% of all bacterial isolates yielded phage particles of varying morphology. The number of lysogenic bacterial isolates was significantly higher in coastal waters as compared to estuarine and saltpan waters. A significant proportion of bacterial isolates were polylysogenic (46%). Our results indicate that viruses are important and very dynamic plankton components in the Northern Adriatic Sea, capable of controlling bacterial population.

JUBILEE ISSUE
FOR THE 50TH ANNIVERSARY
OF THE HUNGARIAN SOCIETY
FOR MICROBIOLOGY
1951–2001

MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

FOR THE 50TH ANNIVERSARY
OF THE HUNGARIAN SOCIETY
FOR MICROBIOLOGY

1951-2001

The Hungarian Society for Microbiology (HMS) was founded in 1951, and its 50th anniversary is celebrated in 2001. The society has a long and distinguished history, and its members have made significant contributions to the field of microbiology. The society's activities include the publication of the journal "Acta Microbiologica Hungarica", the organization of conferences and symposia, and the promotion of research in microbiology. The society's members are scientists, teachers, and students, and they are dedicated to the advancement of microbiology in Hungary and abroad. The society's 50th anniversary is a milestone in its history, and it is a testament to the dedication and hard work of its members. The society's future is bright, and it is confident that it will continue to make significant contributions to the field of microbiology.

HUNGARIAN

PROFESSOR DR. J. KÖRÖSI

KÖRÖSI J.

PROFESSOR

PROFESSOR DR. J. KÖRÖSI

PROFESSOR

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FIFTIETH ANNIVERSARY OF THE HUNGARIAN SOCIETY FOR MICROBIOLOGY

The Hungarian Society for Microbiology (HSM) was founded under the aegis of the Hungarian Academy of Sciences (HAS) in 1951 by the leading microbiologist members of the Academy. In the course of the following years, HSM had worked under the supervision of the HAS. The predecessor of the *Acta Microbiologica et Immunologica Hungarica* (AMIH), the *Acta Microbiologica Acad. Sci. Hung.* Was founded in 1954 by the HAS, – mainly on the initiative of the same members of the HAS mentioned above – as an official journal of the Medical Section of HAS. In the first years, the Presidents of HSM and those of the Editorial Board of the *Acta Microbiologica* had been the same scientists, namely A. Havas then G. Ivánovics, so the cooperation between the two organizations was self-evident.

In 1966, the HSM separated from HAS and joined the Federation of the Hungarian Medical Societies, which is under the supervision of the Ministry of Health, because more than half of its members are physicians. Although AMIH remained the journal of the HAS, the close scientific collaboration did not change between the two organizations. The abstracts of the presentation and many lectures on the many different – national and international – scientific meetings organized by the HSM have been published in the AMIH. Members of the HSM and Council of HSM exerted an intense activity in the Editorial Board of AMIH and mutually, the members of the Editorial Board of AMIH also took part in the work of the Council of HSM. On the basis of these good relations, the Editorial Board and the Council of HSM, as well as their co-workers were invited for commemorial publications. The efforts of the colleagues sending papers to this jubilee issue are highly appreciated. In addition to the scientific publications, a short history of HSM is published and also commemorations of several famous late Hungarian microbiologists without any demand of completeness.

On the occasion of the 50th anniversary of the HSM, we express our highest regard for its successful activity in promoting the development of the different fields of microbiology in Hungary and wish all the best to its future work.

István Nász

Editor-In-Chief of AMIH

Lajos Gergely

President of HSM

50TH ANNIVERSARY OF THE HUNGARIAN SOCIETY FOR MICROBIOLOGY (H.S.M.) (A SHORT HISTORY)*

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In the late forties of the last century the microbiologist members of the then newly re-organized Hungarian Academy of Sciences, namely, *R. Manning*, *G. Ivánovics* and *A. Havas* (*D. Fehér* joined them somewhat later) and the parasitologist academician *S. Kotlán* agreed in the necessity of founding a society of experts working in different fields of microbiology. Thus, the *Hungarian Society for Microbiology* (*H.S.M.*) was founded under the aegis of the *Hungarian Academy of Sciences* in 1951. According to the first constitution of the *H.S.M.* its main task was to advance the following branches of microbiological sciences: general microbiology, virology, bacteriology, immunology, helminthology, acaro-entomology and epidemiology. For this purpose, experts in the fields of human medicine, veterinary medicine, soil research, nutrition research and industry were united in a society (*H.S.M.*) in order to deepen their knowledge and promote the interchange of their experiences and experimental results. Helminthology and acaro-entomology was ruled out in 1964, when the Society of Hungarian Parasitologists was founded.

It was a great advantage for the *H.S.M.* that the first president, *A. Havas*, was at the same time director-general of the *National Institute for Hygiene* (*O.K.I.*), the institution that has played a central role in the organization of the medical application of microbiology in Hungary. *O.K.I.* (and its successor since 1998, the *National Center for Epidemiology*, *O.E.K.*) has intensively supported the activities of the *H.S.M.* during

* This paper was written to commemorate to the fiftieth anniversary of the foundation of the Hungarian Society for Microbiology.

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the past 50 years. *H.S.M.* has been organizing National Microbiological Conferences every year in one of the cities of Hungary and in every fourth year a Microbiological Congress in Budapest with international character. In each of these Congresses 20 or more foreign participants were guests of the *H.S.M.* attempting to return the great hospitality our members enjoyed when they participated as guests in congresses abroad, most frequently in neighbouring countries. The number of participants from abroad has been growing, there were 94 guests from foreign countries at the *13th International Congress of the H.S.M.* organized in 1999 at the *Eötvös Loránd University, Budapest*, by *K. Márialigeti*. In all of these scientific meetings all disciplines of microbiology have been represented and the abstracts of the presentations have been published in the journal *Acta Microbiologica Academiae Scientiarum Hungaricae* (1954–1982), *Acta Microbiologica Hungarica* (1983–1993) and *Acta Microbiologica et Immunologica Hungarica* (from 1994).

The *H.S.M.* joined the *International Association of Microbiological Societies* in 1966 and delegated members into its committees. In 1971, based on the initiative of *I. Dömök* and *E. Farkas*, the *Virology Division* of this Association held the *2nd International Congress for Virology* in Budapest. *G. Berencsi*, *E. Farkas*, *S. Koch*, *I. Hollós*, *E. Molnár*, *G. Nagy* and *P. Ruzicska* made a significant contribution to the organization of this congress. The *International Association of Microbiological Societies* (affiliated to the *International Union of Biological Sciences* as a Division) acquired independence in 1980 and became a Union Member (as the *International Union of Microbiological Societies, IUMS*) of the *International Council of Scientific Unions (ICSU)*. *I. Joó* has been elected as member of the *Council of the International Association of Biological Standardization*, a Committee of *IUMS*.

The *H.S.M.* has also been a member of the *European Society against Viral Diseases* (former name: *European Society against Poliomyelitis*). *I. Dömök* has been member of the presidium of this Society. From 1958 until the elimination of poliomyelitis in Europe, *H.S.M.* had a *Poliomyelitis Committee*, which took part, in cooperation with the mentioned European Society in the organization of the highly successful fight against poliomyelitis in Hungary. The *European Society against Viral Diseases* and the *European Group for Rapid Viral Diagnosis* merged in 1997 and formed a new society, the *European Society for Clinical Virology (ESCV)*. *ESCV* held its *3rd Annual Meeting* in 1999 in Budapest. *G. Berencsi* had the lion's share in the organization of this meeting.

H.S.M. is a national society of *FEMS*, the *Federation of European Microbiological Societies*. Established in 1974, *FEMS* is a Charity and also a Company Limited by Guarantee. *FEMS* provides its journals free of charge for several libraries in Hungary and supports the organization of microbiological congresses and meetings

(Young Scientist Grants, Meeting Support Grants, Workshop Support Grants, Start-Up Funds). It also provides short-term fellowships for young scientists to work in European laboratories and visiting scientist grants to cover travelling costs of senior scientists.

H.S.M. has organized a considerable number of symposia and conferences in which recommendations have been issued in some topics of microbiological nature, having nation-wide importance.

Presidents of the H.S.M.: A. Havas (1951–1954), G. Ivánovics (1954–1958 and 1967–1975), R. Manninger (1958–1967), L. Vácz (1975–1983), J. Mészáros (1983–1993), L. Gergely (1993–present).

Secretary generals of the H.S.M.: E. Farkas (1951–1959 and 1966–1975), G. Weiszfeiler (1959–1966), F. Fornosi (1975–1983), I. Dömök (1983–1988), G. Berencsi (1988–1993), J. Minárovits (1993–present).

In addition to the president and the secretary general, the Presidium of the *H.S.M.* includes the vice presidents (at present I. Béládi and J. Mészáros), the treasurer (I. Lontai at present) and the secretary (at present G. Szűcs), the chairpersons of the Sections (see below) and the chairman of the Controlling Committee of the Society (L. Emődy, from 1998–2003). During the last election (Miskolc, 1998) the General Assembly of the *H.S.M.* elected altogether 41 members and substitute members of the Board and the Control Committee. The Board Meeting held immediately thereafter elected the members of the Presidium.

In the course of years, several scientific sections of *H.S.M.* have been formed. At present, there are 6 sections, namely, *Section of Agricultural and Food Microbiology* (chairmen J. Farkas and M. Kecskés), *Section of Bacteriology* (chairman, L. Emődy), *Section of Immunology* (chairman, S. Tuboly), *Section of Industrial Microbiology* (chairman, A. Szentirmai), *Section of Mycology* (chairperson, A. Maráz), *Section of Virology* (chairman, G. Berencsi).

Among the Sections, the *Section of Immunology* has been the most active. Its activity extended to all fields of immunology (initially even allergology) till 1971. Then the *Hungarian Society for Immunology* was founded, and since then the activities of the Section of Immunology have only covered immunity against infectious diseases, problems of vaccinations and standardization of immunological procedures.

H.S.M. had worked under the supervision of the *Hungarian Academy of Sciences* until 1966. Then *H.S.M.* joined the *Federation of the Hungarian Medical Societies* (MOTESZ), which is under the supervision of the *Ministry of Health*. This change was carried out, because somewhat more than half of our members are physicians. Of course, microbiologists of other professions, among them veterinarians, biologists and engineers, are always represented in our presidium and committees. The

constitution of *H.S.M.* has been modified several times (the latest change was in 1989) and *H.S.M.* was registered as a social organization in 1989 (Budapest Court registration No. 590).

Members of the *H.S.M.* exerted an intense activity in the Editorial board of *Acta Microbiologica Acad. Sci. Hun.*, *Acta Microbiologica Hungarica* and *Acta Microbiologica et Immunologica Hungarica*. Editors-in-chief: A. Havas (1954), G. Ivánovics (1955–1976), B. Lányi (1976–1981), and I. Nász (1982–present).

H.S.M. has always supported microbiologists to attend scientific meetings in this country and abroad. In order to facilitate the achievement of this goal and to facilitate the development of research in microbiology in Hungary, the *Foundation of the Hungarian Society for Microbiology* was created in 1989. The *Foundation* was registered in the same year (Budapest Court registration No. 460). The deed of foundation of the *Foundation of the H.S.M.* was modified in 1997 to include a statement that the *Foundation* is independent of political parties, it does not receive support from them and neither nominates nor supports candidates for Parliament.

It became a tradition of our *Society* that it offers prizes for the best presentations held by young scientists at our yearly conferences, congresses. In addition, *H.S.M.* founded the “*Manninger Rezső Award*” in 1974 for outstanding members of the *Society* having internationally acknowledged scientific activities and being active in the life of our *Society*.

List of members with Manninger Award

	Name	Profession	Date
1.	György Ivánovics	physician	1974
2.	Béla Johan	physician	1974
3.	János Köves	veterinarian	1974
4.	István Nyirédy	veterinarian	1974
5.	Zoltán Alföldi	physician	1974
6.	Gyula Weiszfeiler	physician	1974
7.	Elek Farkas	physician	1975
8.	Károly Rauss	physician	1975
9.	László Erdős	physician	1976
10.	György Habán	physician	1976
11.	Lajos Vácz	physician	1977
12.	Tamás Szent-Iványi	veterinarian	1978
13.	Gyula Takátsy	physician	1979

14.	Károly Vas	chemical engineer	1980
15.	Ferenc Fornosi	physician	1980
16.	János Mészáros	veterinarian	1981
17.	István Joó	physician	1981
18.	István Nász	physician	1982
19.	Zoltán Klement	agricultural engineer	1982
20.	István Földes	physician	1983
21.	István Szabó	physician	1983
22.	Iván Kétyi	physician	1983
23.	Ilona Béládi	physician	1984
24.	Béla Lányi	physician	1984
25.	Adorján Bartha	veterinarian	1985
26.	Ferenc Kemenes	veterinarian	1986
27.	Mihály Kecskés	biologist	1986
28.	József Szita	physician	1987
29.	Ilona Szeri	physician	1987
30.	István Kiss	chemical engineer	1988
31.	Mrs Nyerges Gáborné	physician	1988
32.	Lajos Ferenczy	biologist	1989
33.	István Dömök	physician	1989
34.	Lajos Alföldi	physician	1990
35.	László Stipkovits	veterinarian	1990
36.	Tibor Deák	biologist	1991
37.	Sándor Tuboly	veterinarian	1991
38.	Hedda Milch	physician	1992
39.	Attila Szentirmai	biologist	1992
40.	József Farkas	chemical engineer	1993
41.	Erzsébet Molnár	physician	1993
42.	Ervin Novák	biologist	1993
43.	György Berencsi	physician	1994
44.	Béla Nagy	veterinarian	1994
45.	Erzsébet Nagy	physician	1995
46.	József Molnár	physician	1996
47.	László Hornok	biologist	1997
48.	Rozália Pusztai	physician	1997
49.	Ferenc D. Tóth	physician	1998
50.	Levente Emödy	physician	1998
51.	János Varga	veterinarian	1998

52.	József Földes	physician	1999
53.	Anna Maráz	biologist	2000
54.	Yvette Mándi	physician	2000
55.	Éva Ádám	pharmacist	2001
56.	Éva Gönczöl	physician	2001

For promoting the international relationships of the *Society* a number of distinguished foreign microbiologists were chosen to honorary members of *H.S.M.* This honorary membership was given to microbiologists, who had strong working relations with some of our microbiological institutions and have made efforts to participate in the activities of our Society.

Summarizing the results of the last 50 years, we are of the opinion that the *Hungarian Society for Microbiology* fulfilled its main task and was successful in promoting the development of microbiology in Hungary. This is the more important, as sciences (including and especially microbiology) did develop in such a fantastic tempo, that has been never witnessed in the history of mankind. Thus, we are looking forward with curiosity to new scientific challenges and we are optimistic about the coming of the next 50 years.

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A TRIBUTE TO THE FIRST FOUR PRESIDENTS OF THE HUNGARIAN SOCIETY FOR MICROBIOLOGY*

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" Knowledge itself is power "
(Francis Bacon)

The Hungarian Society for Microbiology is currently celebrating the 50th anniversary of its foundation. On this occasion, we remember the first four Presidents of the Society, whose activities led to the Society becoming a thriving community covering all branches of microbiology.

Three of the first four Presidents of the Hungarian Society for Microbiology, András Havas, György Ivánovics and Rezső Manninger, were elected Members of the Hungarian Academy of Sciences. This did not mean only that they were recognized as outstanding microbiologists in Hungary: the support provided by the Academy furnished them with an opportunity to increase the level of research and diagnostic work and education in microbiology, a possibility which was not generally available during the 1950s and 1960s. One of the first four Presidents, Lajos Váczi, was not elected Member of the Hungarian Academy of Sciences, his credits, however, greatly contributed to the relatively rapid development of microbiology during the post-war years in the politically and economically depressed Hungary. President Lajos Váczi was involved in the organization of the microbial laboratory work in the country after the Second World War. His unquestionable merit in the education of young microbiologists is proved by the fact that, at present, three of his disciples occupy the chairs at the Departments of Microbiology at three of the four Medical Universities in Hungary.

* This paper was written to commemorate to the fiftieth anniversary of the foundation of the Hungarian Society for Microbiology.

The first President of the Society was András Havas, from 1950 to 1954. **András Havas** (1891–1954) was born in Debrecen. He obtained his medical diploma at the University of Kolozsvár. After the First World War he emigrated first to Austria and then to the Soviet Union because of the political situation. From 1927 to 1946, he worked at the Central Research Institute for Tuberculosis in Moscow, where he was awarded his C.Sc. for studies on *Mycobacterium tuberculosis* in 1938.

He returned to Hungary in 1946 and joined the staff of the National Institute of Hygiene in Budapest. He became chairman of the Department of Tuberculosis and his main interest then turned to the organization of BCG vaccination in Hungary. From 1949 until his death, he was Director of the National Institute of Hygiene. In this position he continued his activities relating to BCG vaccination. His intention was to introduce the BCG vaccination of neonates in Hungary. He spared no efforts to create the personal and objective conditions for diagnostic and experimental work in his Institute. He was an openhearted person with deep social concerns. He was elected a Corresponding Member of the Hungarian Academy of Sciences in 1949. He was an active member of the Medical Section of the Academy and a member of many societies dealing with medicine and public health. The Hungarian Society for Microbiology was created on his initiation and he was keen to found the *Acta Microbiologica* edited by the Publishing House of the Hungarian Academy of Sciences where Hungarian microbiologists would have the possibility to publish their results. Different state awards acknowledged his outstanding activities in public health.

György Ivánovics was President of the Hungarian Society for Microbiology from 1954 to 1958 and then again from 1967 to 1975. **György Ivánovics** (1904–1980) was born in Budapest. He received his medical training at Péter Pázmány University in Budapest and was awarded his medical diploma in 1928. He was appointed chairman of the Institute of Microbiology at the Medical University of Szeged in 1940, and he remained in that position until 1974. He spent several longer periods abroad. He was the first biotechnologist in Hungary to introduce tissue culture work in his Institute, in the early 1950s.

With his chemist colleague Győző Bruckner, he discovered that the antiphagocytic capsule of *Bacillus anthracis* is composed of poly-D-glutamic acid. He made many basic observations concerning megacin, the bacteriocin of *B. megaterium*. His major interest was and remained until his retirement the lysogenicity of *B. megaterium*. He was elected a Corresponding Member of the Hungarian Academy of Sciences in 1945. He created the "Ivánovics school" in microbiology in Hungary: of his close students or colleagues, two became Members of the Hungarian Academy of Sciences, four professors in microbiology, and two chief doctors in laboratories at hospitals in Hungary. He was a member of many Hungarian and foreign scientific

societies. He was one of the founders of the Hungarian Society for Microbiology. He authored or co-authored more than 200 publications, most of which appeared in internationally recognized journals. He received a number of honors, among them the Kossuth Prize twice (1948 and 1952). His influence was pervasive. Because of his energetic and unflagging devotion to the advancement of microbiological research in Hungary, he was greatly respected and admired by his disciples and colleagues. Those who trained, worked and served with him warmly remember his unrelaxing enthusiasm for research work and his great appetite for life.

Rezső Manninger was President of the Hungarian Society for Microbiology from 1958 to 1967. **Rezső Manninger** (1890–1970) was born and attended school in Sopron. He then studied at the Veterinary College in Budapest, where he graduated in 1912. He next took up a position on the staff of the Institute of Epidemiology at the Veterinary College, chaired by the internationally recognized Professor Ferenc Huttyra. For more than 50 years, Dr. Manninger was working in this Institute, and from 1927 until 1963 he served as chairman there. In 1927 he was elected a Corresponding Member, and in 1935 a Member of the Hungarian Academy of Sciences.

He devoted his whole life to Hungarian veterinary medicine. He was keen to develop veterinary practice, education and science to the European level. He was the first veterinary doctor to serve as Vice-President of the Hungarian Academy of Sciences. By virtue of his extraordinary professional knowledge and very human personality, he was President of the Hungarian Society for Microbiology for 10 years. Everybody liked him and he may be stated to have had not even one enemy. He retired from the Institute of Epidemiology in 1963. He was an honorary member of many foreign Academies and Honoris Causa doctor of many foreign Veterinary Universities and Colleges. He received a number of honors among them the Kossuth Prize twice. He published 272 papers and was author or co-author of 26 books dealing with veterinary medical problems. His textbook (F. Huttyra, J. Marek und R. Manninger: *Spezielle Pathologie und Therapie der Haustiere*, Gustav Fischer Verlag, Jena, 1938) was internationally recognized and translated into English, Italian, Russian, Polish and Chinese. He had a charming personality and charisma. He will be remembered as a person whom everybody liked, and no one was respected and admired with greater consistency than he was.

Lajos Váczi was President of the Hungarian Society for Microbiology from 1975 until 1983. **Lajos Váczi** (1917–2000) was born in Komádi. He graduated from the Medical University of Debrecen in 1942, "Sub auspiciis gubernatoris". He worked in the Institute of Hygiene at the Medical University of Debrecen until 1944. From 1945 to 1958 he was in Budapest, first as head of the Professional Teaching Department at the Ministry of Health. From 1951 he became a member of the

Department of Bacteriology at the National Institute of Hygiene, and between 1956 and 1958 he was head of this Department. In 1958 he was appointed chairman professor at the Institute of Microbiology of the Medical University of Debrecen. He received his D.Sc. from the Hungarian Academy of Sciences in 1968. He was author or co-author of 206 scientific papers. His textbook was an interesting experiment for the introduction of "organ-specific microbiology" into medical education. He was one of the founders of the Hungarian Society for Microbiology. He was an honorary member of many foreign universities and international societies. He initiated the professional career of several young talented microbiologists. It is due to him that research in virology started in the Institute of Microbiology in Debrecen. The topics of study were human herpes viruses and, for the first time in Hungary, tumor-causing viruses. His disciples continue to work with human herpesviruses and papillomaviruses and their activities are internationally recognized.

FACTORS AND FACTS IN HUNGARIAN HIV/AIDS EPIDEMIC, 1985–2000*

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In Hungary among others there were some special factors, which shaped the outcome of HIV/AIDS epidemic. (1) In the early period of pandemic the “iron curtain” delayed and limited the importation of HIV to Hungary. (2) In 1985, at the time of detection of first HIV infected persons the etiological diagnostic tools were already commercially available and laboratory facilities have been created immediately for HIV antibody tests in networks of blood banks, public health and venereological services. (3) Laboratory facilities together with introduced health regulations resulted in (a) elimination of possibility of nosocomial HIV transmission by blood, blood products and organ transplantation; (b) efficient case finding and contact tracing in population groups potentially playing a significant role in spreading of infection; (c) opportunities for voluntary HIV testing free of charge. (4) Broad scale education and information activities have been developed from the beginning by governmental and non-governmental organizations alike. (5) Parenteral drug abuse did not play a role in spreading of HIV, so far.

The above factors resulted in a slowly developing moderate epidemic. The facts are as follows. By the end of 2000 altogether 879 HIV positive (666 male, 100 female and 113 anonymous) persons have been notified, 377 (344 male and 33 female) of whom showed already the characteristic features of AIDS and 229 died. 29% of registered HIV positive persons have been foreigners originating from 56 countries. The cumulative incidence rate of AIDS was 38 per million population. 73% of Hungarian HIV positive persons and 72% of patients with AIDS belonged to transmission group of men having sex with men. The age of HIV positive persons at the time of detection was between 20 and 49 years in 81% and 72% of them resided in or around Budapest.

Keywords: delayed HIV importation, early measures, low HIV/AIDS cumulative incidence rates

* This paper was written to commemorate to the fiftieth anniversary of the foundation of the Hungarian Society for Microbiology.

Data on HIV/AIDS situation in different countries regularly published by the WHO, UNAIDS and European Centre for the Epidemiological Monitoring of AIDS [e.g. 1, 2, 3] like a number of Hungarian and other articles [e.g. 4, 5, 6] unanimously indicated that since 1985 Hungary have experienced a moderate HIV/AIDS epidemic with low annual and cumulative incidence rates. There were some publications, which mentioned also certain reasons for the favourable situation [7, 8]. Nevertheless a complete analysis of factors playing a role in and their effects on the course of epidemic have not yet been published. The aim of this review is to give account of all the factors and present the facts reflected by the epidemiological characteristics.

Special and common factors

It is well known that till the end of 1989 a constructed and legislative "iron curtain" existed in Hungary which made difficult for Hungarians to travel abroad especially in the early period of HIV/AIDS pandemic. At the same time it discouraged citizens of Western Hemisphere to come to Hungary either as tourists or as visitors looking for special amusements. That situation did not prevent, nevertheless certainly delayed and limited the importation of HIV to Hungary. This is why the first HIV infected persons could be detected only in 1985 and the first AIDS case in December 1986.

It was an advantage that when the spreading of infection became known in 1985 the laboratory diagnostic tools were already commercially available. Thus based on recommendation of a small expert group (which served for basis later on for the National AIDS Committee) the authorities immediately decided to allocate appropriate budget to create laboratory facilities for HIV antibody tests and surveys in networks of Blood Banks, of Public Health Services and of STD Outpatient Clinics. So that from July 1986 all the blood donations have been tested compulsorily for HIV in the Blood Banks and the alternative laboratories have also been ready to carry out the needed tests. Originally 36 laboratories were equipped in the network of Blood Banks and 12 in the other health services. One confirmatory laboratory has served for the Blood Banks and another one for the alternative laboratories. The number of laboratories has changed later on but the original rule – that only designated, accredited laboratories were authorised to carry out HIV tests – remained unchanged. There have been 146 establishments nation-wide for collection of blood samples for HIV tests including 3 counselling places where tests could anonymously be requested. In addition physicians in the health care service have been authorised to take blood samples for diagnostic

HIV tests if it could be suspected that the actually observed ill-condition was related to HIV infection.

In September 1985 a Ministerial Decree pronounced AIDS as a compulsorily notifiable disease and ordered to admit all the patients with suspected AIDS to the Central Hospital for Infectious Diseases, Budapest. Since 1986 the HIV tests have been made available for anybody free of charge.

In 1986 a methodical guideline signed by the State Secretary of Health and later in 1988 a Ministerial Decree entitled "Measures aimed at prevention of spreading of AIDS" has introduced the compulsory HIV tests for biological donations (blood, organs, tissues and seminal fluid) as well as for persons belonging to certain population groups which potentially may play a significant role in transmission of infection. These have been as follows. (1) Partners of detected HIV positive persons and those who might have been exposed to infection by them (e.g. newborns). (2) Patients attending STD Clinics. (3) Prostitutes arrested for an offence related to soliciting. (4) Prisoners on admission to detention centres. (5) Inmates of correction homes for adolescents. (6) Uncovered intravenous drug abusers.

Anonymous HIV tests have been made possible since 1988. Recent regulations ordered name based report of the cases with confirmed positive test results.

The following considerations served for basis of the above-mentioned regulations. In Hungary from the beginning HIV/AIDS has been viewed as a crisis in public health that had some important human right dimensions and not as a crisis in human rights that had some public health dimensions [9]. In agreement with Angell's opinion [10] it has been the basis of regulations that epidemiological problems should be distinguished from social ones and dealt with both simultaneously but separately. Infection control can only be made by epidemiological measures based on the knowledge of characteristics of given infection whilst social problems can only be solved by educational, legal and economic means. Based on this opinion health authorities have the duty to make efforts to break the chain of transmission of infection by all possible legal means. Simultaneously every kind of discrimination and stigmatisation has to be prevented and the strictest rules of medical confidentiality to be ensured. Protection should be provided both to HIV negative people – i.e. protection against infection – and to HIV positive people – i.e. protection against discrimination [11]. The latter being a social problem should be addressed accordingly.

From epidemiological point of view it is justified to make efforts for early detection of infected persons since it is the interest both of the infected individual and of the whole community.

Early identification of infection certainly results in benefits of the individual person because medical care and regular follow-up can be instituted (a) which gives an

opportunity to induce personal motivation for healthier life style and to avoid abuses as well as activities affecting the pathological processes; (b) which creates opportunity in time for highly active anti-retroviral therapy (HAART) resulting in longer symptom-free life and in prolonged survival; and (c) which can ensure the treatment of opportunistic infections as soon as they occur.

Early detection of infection and its appropriate management results in benefits to community as the probability of transmission of HIV infection can be reduced. (a) It is certain that personal counselling for the patients and for their partners leads to better motivation for safe sex behaviour than the general health promotional programmes in media. (b) HAART diminishes the viral load in the blood and in genital secretions, thus the likelihood of HIV transmission by blood and seminal or vaginal fluids will be diminished.

A wide range of HIV/AIDS prevention and control activities has been carried out since the establishment of AIDS programme. Intensive training of health care workers has been started already in the early period by publications, methodical guidelines [e.g. 12–15], and by lectures. Broad scale education, information activities have been developed for the general population, and for special population groups (e.g. for men having sex with men, for teenagers, for prostitutes and their clients, for prisoners, for immigrants, for tourists, for drug users and for HIV positive persons). In 1987 e.g. 4 million leaflets have been posted to Hungarian families dealing with the most important information on the virus, on its infection and on the preventive possibilities. In these activities both governmental and non-governmental organisations have taken part involving also the media.

It is certain that in recent years the illicit drug use has increased especially among adolescents and young adults. There are no signs, however, that spreading of HIV would have begun by parenteral drug abuse in Hungary, so far. Most recently strict regulation has been enacted to restrain the drug abuse.

Epidemiological facts

The first HIV positive persons were detected in 1985 and by the end of 2000 their number increased to 879 (Fig. 1). 766 of them have been registered with an identification code and 113 remained anonymous. It is of note that some of the anonymous persons are certainly included also in the coded group. The annual mean of newly detected coded HIV positive persons was 48 and their annual number varied between 16 and 74. The 113 anonymous HIV positive persons were uncovered during a 9-year period between 1989 and 1997. Their annual main number was 13. Among the

coded 475 HIV positive persons registered between 1985 and 1995 altogether 305 (64%) had been detected in connection with compulsory screening partly of those attending the STD Clinics and partly of partners of HIV positive patients [16].

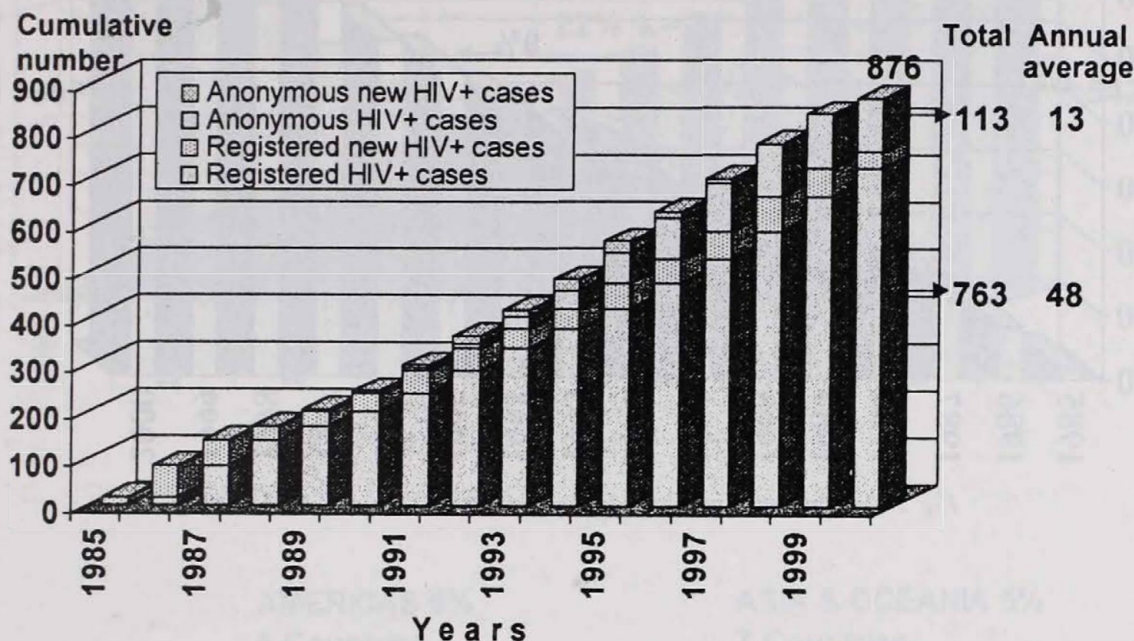


Fig. 1. Cumulative number of detected anonymous and registered HIV positive persons by years, 1985–2000

666 of 766 registered HIV positive patients were males (87%) and 100 females (13%) (Fig. 2). Nevertheless the rate of female HIV positive persons was only 6% in 1990 and 9% in 1995. Thus a significant increasing tendency could be observed in rate of female patients in the last 10 years.

Foreign citizens have been in considerable number among the HIV positive persons registered in Hungary (Fig. 3). By the end of 2000 altogether 221 foreigner HIV positive persons originating from 56 countries were notified representing 29% of total number of registered HIV positive subjects. 49% of foreigners originated from 19 European, 37% from 25 African, 9% from 5 American, 5% partly from Asian (5) and partly from Pacific (2) countries (Fig. 4).

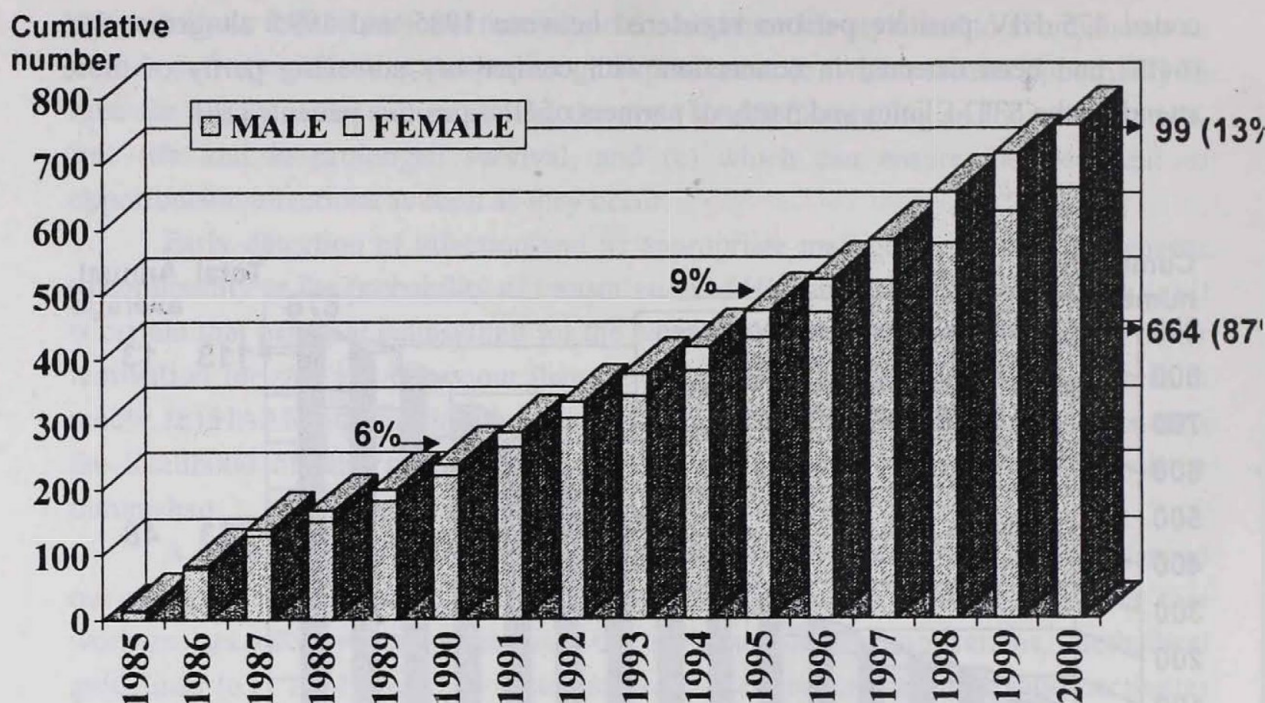


Fig. 2. Distribution of registered HIV positive persons by sex, 1985–2000

HIV infection of these persons was detected in connection with applications for university fellowships (especially submitted before 1990 by Africans), or for long-term and permanent residence as well as for repatriation. A number of them, however, came to Hungary directly for HIV testing owing to lack or difficulties in testing possibilities in their home countries (e.g. 50 HIV positive persons from Romania).

Analysing the transmission categories of registered 545 Hungarian HIV positive patients, it is clear that vast majority of them belonged to group of men having sex with men (Fig. 5). 395 of 545 HIV positive persons (73%) registered between 1985 and 2000 belonged to that risk group. There was, however, a significant decrease in rate during the recent years. In the period between 1996 and 2000 homo-/bisexual men represented 65% of registered Hungarian HIV positive patients in contrast to the former periods when their rates attained 76% (1985–1990) and 79% (1991–1995), respectively. Opposite tendency could be observed in cases with heterosexual transmission. The rate found between 1985 and 1990 was only 5%, whilst that between 1996 and 2000 attained 26%. In the early period of epidemic all the known 629 haemophiliacs had been tested for HIV and 28 of them proved to be positive (4.5%) representing 14% of HIV positive patients registered between 1985 and 1990.

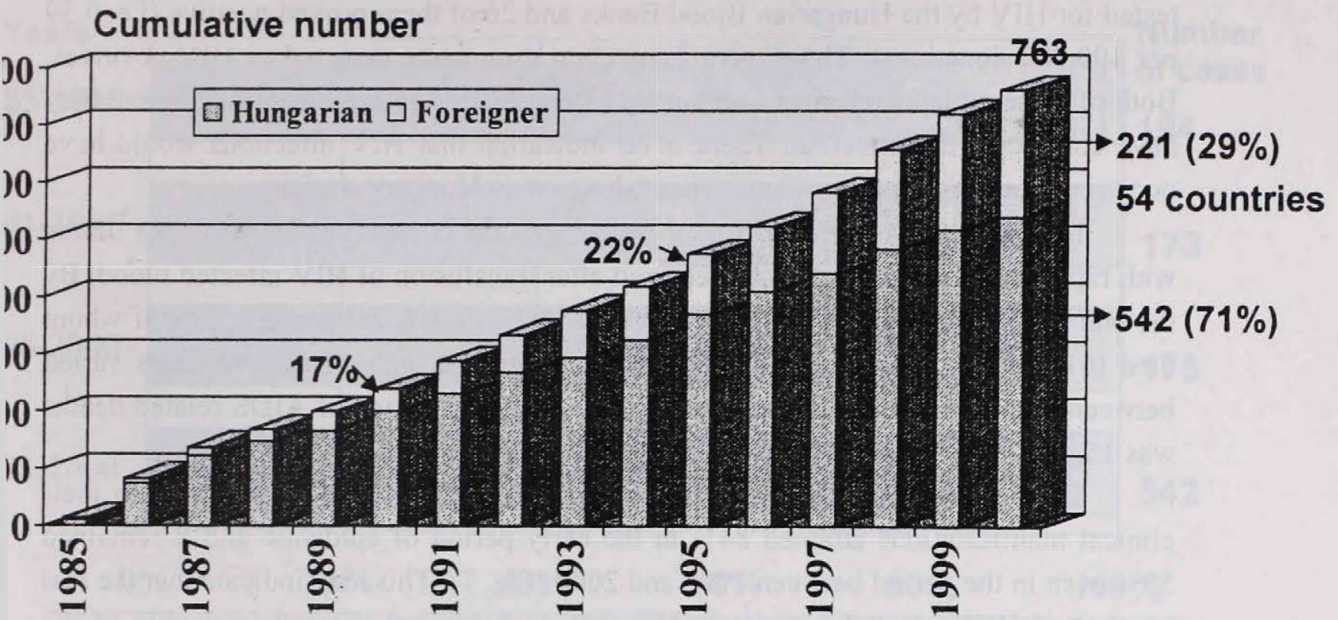


Fig. 3. Citizenship of registered HIV positive persons, 1985–2000

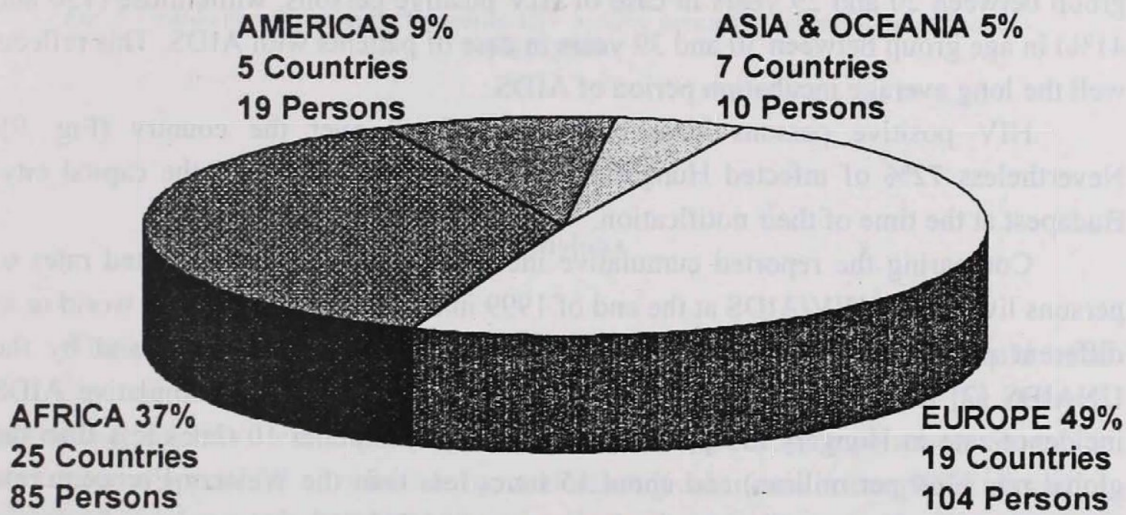


Fig. 4. Distribution of HIV positive foreigners registered between 1985 and 2000 in Hungary by geographic location of their home countries

Since that time no new HIV positive cases have been found among the patients with haemophilia due to the fact that all the imported HIV positive clotting factor concentrates had been destroyed at the end of 1985 and since that only safe products have been administered. From 1986 to 1999 altogether 6,645,000 blood donations were

tested for HIV by the Hungarian Blood Banks and 26 of them proved positive (i.e. 0.39 per 100,000 donations). There were 2 injecting drug users detected as HIV positives. Both of these patients returned to Hungary after a long time period abroad, where they have contracted the infection. There is no indication that HIV infections would have occurred as a consequence of intravenous drug use in Hungary, so far.

In Hungary the first AIDS case was diagnosed in December 1986. That illness with rapid deterioration and death occurred after transfusion of HIV infected blood. By the end of 2000 altogether 377 patients with AIDS have been reported, 229 of whom died (61%) (Fig. 6). Since 1987, the annual number of newly reported cases varied between 7 and 46 with an annual mean of 25. Annual average of AIDS related deaths was 15.

Rate of patients with AIDS whose HIV infection was detected prior to their clinical manifestations attained 84% in the early period of epidemic and it remained 59% even in the period between 1997 and 2000 (Fig. 7). This may indicate that the real number of HIV infected patients in Hungary probably not more than double of the detected one.

81% of HIV positive and 79% of AIDS patients belonged to age groups between 20 and 49 years (Fig. 8). The highest number and rate (258 and 34%) occurred in age group between 20 and 29 years in case of HIV positive persons, while those (150 and 41%) in age group between 30 and 39 years in case of patients with AIDS. This reflects well the long average incubation period of AIDS.

HIV positive persons have been detected all over the country (Fig. 9). Nevertheless 72% of infected Hungarians were living in or around the capital city, Budapest at the time of their notification.

Comparing the reported cumulative incidence rates and the estimated rates of persons living with HIV/AIDS at the end of 1999 in Hungary to those in the world or in different parts of Europe based on data published by the WHO [17] and by the UNAIDS [2] the following results can be obtained (Table 1). The cumulative AIDS incidence rate in Hungary (35 per million population) is about 10 times less than the global rate (369 per million) and about 15 times less than the Western-European rate (532 per million). According to the estimation of UNAIDS the number of persons living with HIV/AIDS in the world was 34.3 million at the end of 1999, which represented a global rate of 5756 per million population. Number of Hungarians living with HIV/AIDS at the same dates was estimated by the UNADS to 2500, i.e. representing a rate of 248 per million, which is about 23 times, less than the global rate.

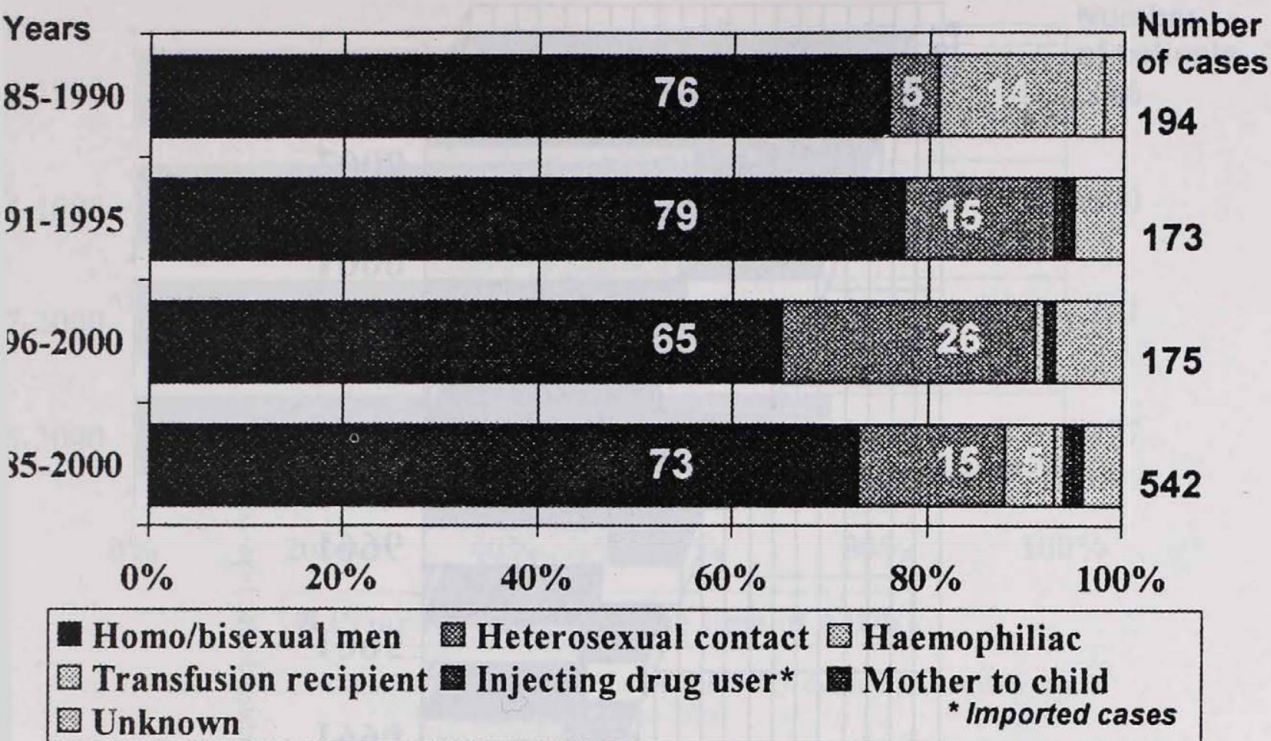


Fig. 5. Distribution of registered Hungarian HIV positive persons by transmission categories, 1985-2000

Conclusions

The presented data clearly indicate that the HIV/AIDS epidemic could have been kept on a moderate level in Hungary. Special factors such as the delayed importation of HIV, early possibilities of HIV diagnostics, regulations aimed at prevention of spreading of infection by epidemiological means with special regard to individual interests and confidentiality as well as lack of spreading of HIV by intravenous drug use together with certain common factors significantly contributed to the remarkably good HIV/AIDS epidemiological situation. The Hungarian practice was criticised repeatedly in the former years but the epidemiological facts indicate that the policy and programmes have been well oriented and effective, thus they rightly give incitement to continue the activities on the same lines.

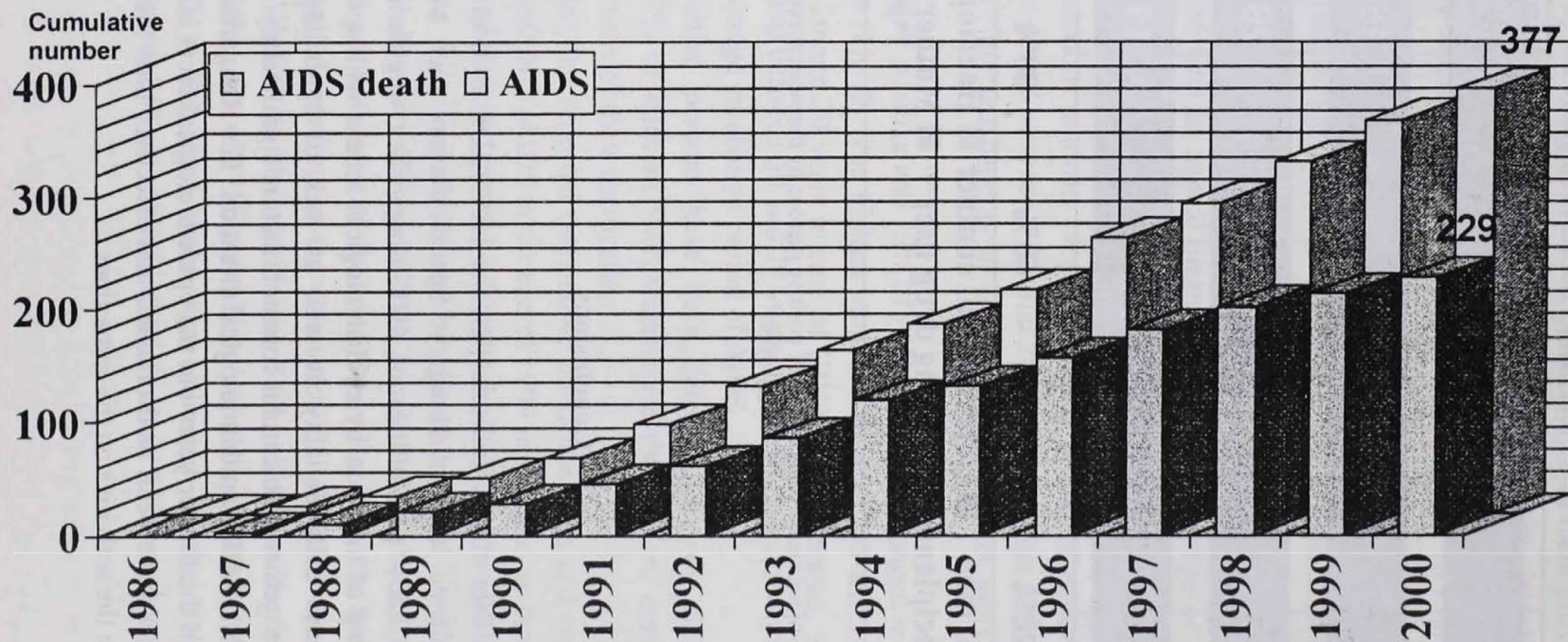


Fig. 6. Registered AIDS cases and AIDS related deaths, 1986–2000

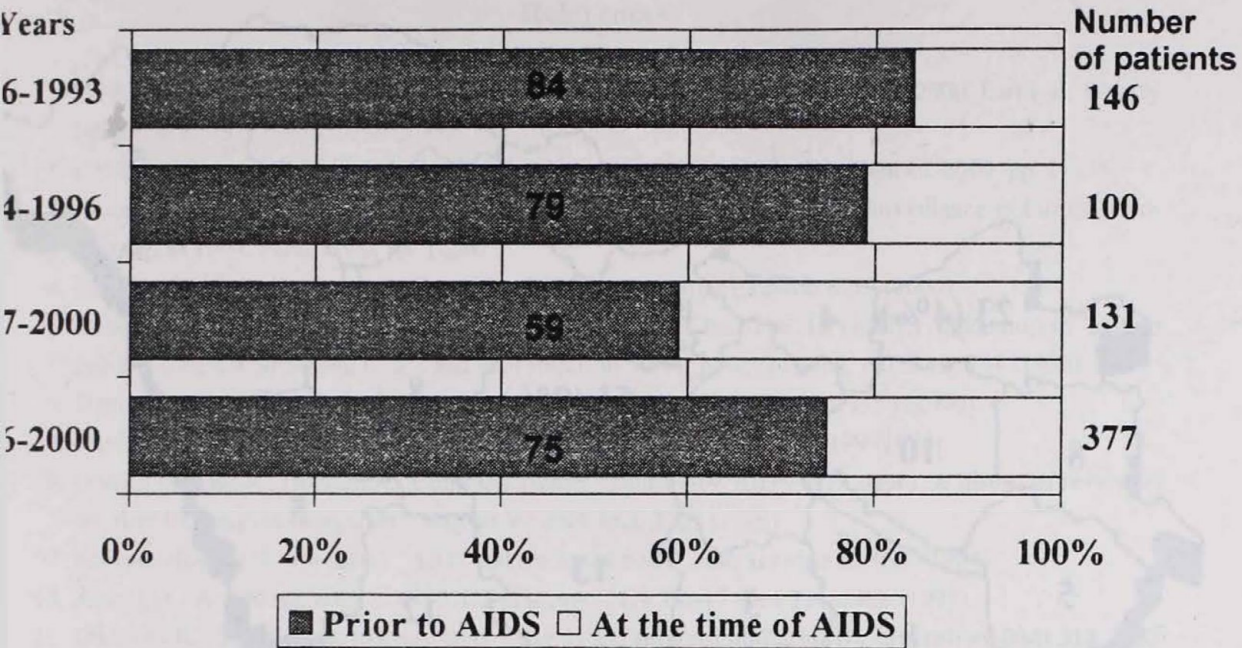


Fig. 7. Rates of patients with AIDS (%) whose HIV infection has been detected prior to their clinical manifestations, 1986-2000

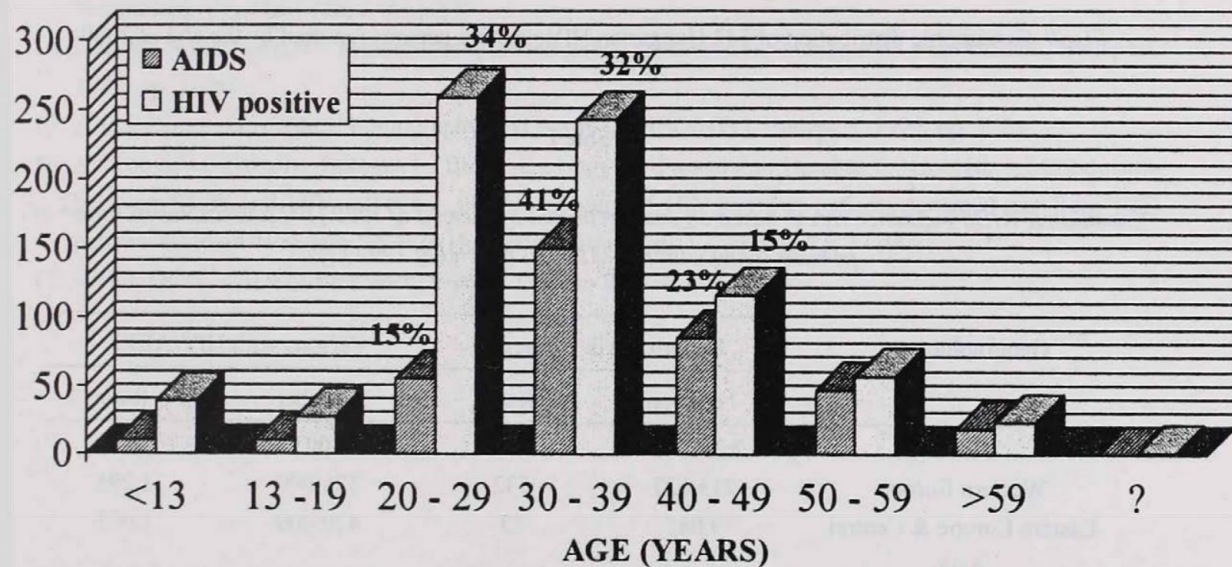


Fig. 8. Distribution of registered HIV positive persons and of AIDS cases by their age at the time of notification.

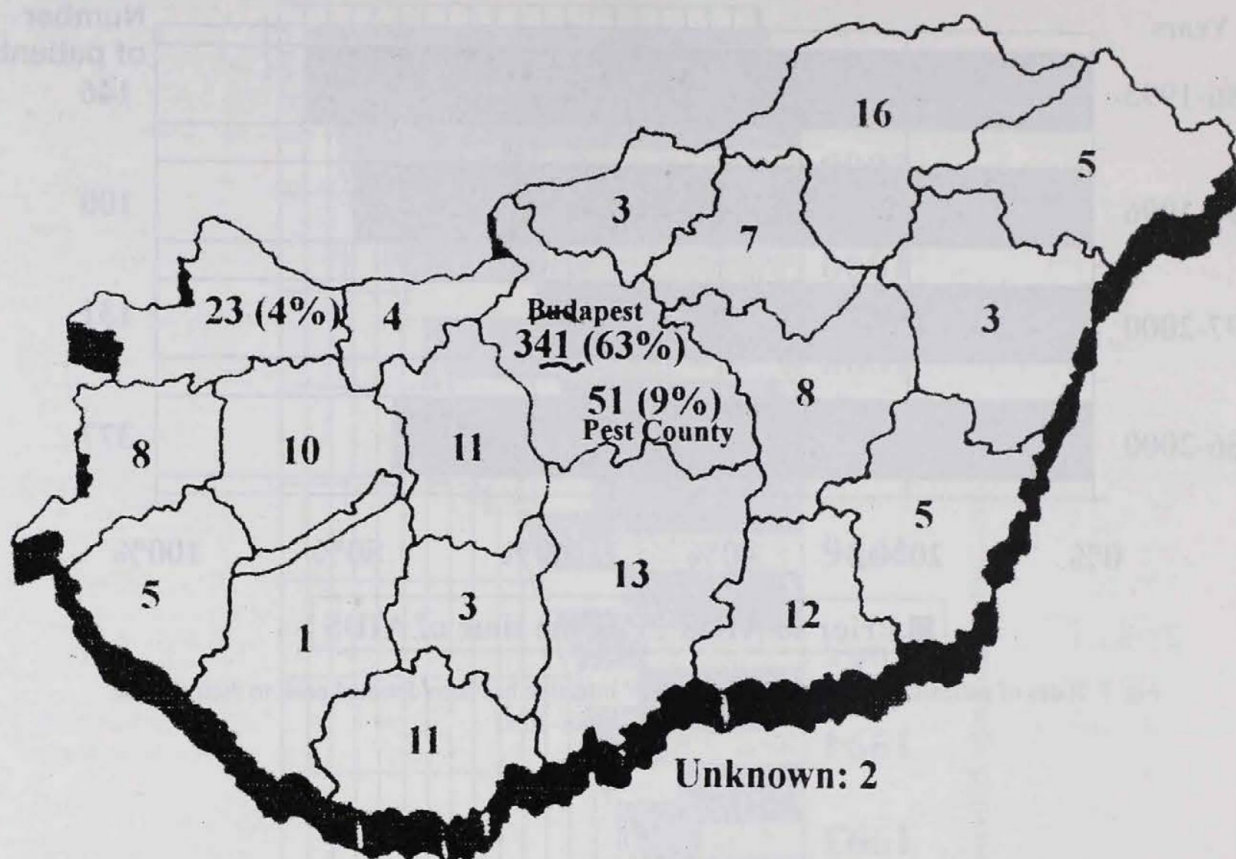


Fig. 9. Geographic distribution of 542 Hungarian HIV positive persons reported by the end of 2000

Table I

Cumulative AIDS incidence rates based on cases reported by the end of 1999 [17] and estimated rates* of persons living with HIV/AIDS at the end of 1999 [2]*

Geographic area	Reported AIDS cases		Persons with HIV/AIDS	
	Number	Rate	Number	Rate
Global	2,201,461	369	34,300,000	5,756
Western Europe	213,627	532	520,000	1,295
Eastern Europe & Central Asia	9,085	23	420,000	1,073
Hungary	350	35	2,500	248

*per million of population

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HUMAN CYTOMEGALOVIRUS LOAD IN THE PERIPHERAL BLOOD DETERMINED BY QUANTITATIVE COMPETITIVE POLYMERASE CHAIN REACTION*

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Primary human cytomegalovirus infection and the viral reactivation from latency are major complications in organ transplant recipients. In the peripheral blood the replicating virus can be detected either by nucleic acid based tests or by demonstrating the HCMV structural proteins in antigenemia test. We developed a quantitative competitive PCR method to assess the HCMV load in the peripheral blood. The viral load in nine healthy blood donors and in four renal transplant recipients with negative antigenemia test was in the same range: 5–124 (median: 18) HCMV copies/ 10^6 β -globin copies for healthy blood donors and 16–48 (median: 37) HCMV copies/ 10^6 β -globin copies for the transplant recipients. Three antigenemia positive renal transplant recipients had a HCMV load of $2.2 \times 10^5/10^6$ β -globin, $1.5 \times 10^4/10^6$ β -globin and $6.5 \times 10^3/10^6$ β -globin, respectively. In conclusion, the quantitative measurement of HCMV load in the peripheral blood correlated well with the routine HCMV antigenemia test. The DNA-based test, however can detect earlier the reactivation of the HCMV infection.

Keywords: cytomegalovirus, renal transplant recipients, quantitative PCR

* This paper was written to commemorate to the fiftieth anniversary of the foundation of the Hungarian Society for Microbiology.

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Introduction

Primary human cytomegalovirus (HCMV) infection and the viral reactivation from latency are major complications in organ transplant recipients. After the primary infection, the virus establishes a latent infection in certain cell types like monocytes and CD 34+ bone marrow cells [1, 2]. During the latent stage, the number of mononuclear cells carrying the virus is extremely low (about 1/10,000) in the peripheral blood [3] and the HCMV DNA can be detected neither in the polymorphonuclear leukocytes (PMNLs) nor in the plasma [1, 4, 5]. Regarding viral expression during latency, only the immediate early (IE) region is transcribed at a reduced rate [4]. The differentiation of monocytes into tissue macrophages enables the lytic gene expression of HCMV and the release of infectious virions. However the cells expressing the lytic genes of HCMV are controlled by the HCMV specific cytotoxic T-lymphocytes in the immunocompetent host [6]. Thus, immunosuppression is a risk factor for the HCMV viraemia and the HCMV diseases. During the viraemic phase, the complete viral expression is detected in the peripheral blood mononuclear cells [7, 8]. The polymorphonuclear cells may take up the virus and may reveal even the expression of the IE region [9]. The diagnosis of acute HCMV infection is based on the demonstration of virus replication. Detection of anti-HCMV IgM type antibodies in organ transplant recipients has a limited diagnostic value, because active HCMV infection can become fatal even before the IgM type antibodies appear. The HCMV antigenemia test demonstrates the presence of the pp65 tegument protein in the circulating leukocytes. The pp65 is the most abundantly produced viral protein in early and late phases of the viral replication. Since the viral genome can be detected in low copy number in the latent phase of the infection, DNA based detection of HCMV viraemia from clinical samples containing mononuclear cells (e.g. whole blood) requires quantitative measurement of the viral load [10]. Alternative methods to examine the virus reactivation are the detection of early-late mRNA expression [11] and detection of viral genome in the plasma phase [12]. We developed a quantitative competitive PCR method using mutant sequences of extended length as internal controls. The results obtained by this methodology from peripheral blood samples of renal transplant patients correlated well with those of the pp65 antigenemia test.

Materials and methods

Cell cultures and clinical samples

Human fibroblast cells were grown in DMEM + 5% fetal calf serum and were infected with human cytomegalovirus (strain AD 169) at a multiplicity of infection 1. Infected cells were grown until 50% of the cells revealed cytopathic effect. The infected and mock-infected human fibroblast cells were harvested and resuspended in 2 ml TNE (10 mM Tris, 100 mM NaCl, 1 mM EDTA) for DNA extraction. The study group consisted of seven renal transplant recipients and nine healthy blood-donors. Peripheral blood samples of the renal transplant recipients were tested for HCMV pp65 antigenemia with CINAkkit Rapid Antigenemia test (Argene Biosoft, Varilhes France). Leukocytes for both the antigenemia and the PCR testing were isolated from 1,5 ml heparinized blood by lysing erythrocytes in eight volumes ice-cold lysis solution (0.085% NH_4Cl). After washing once more in the lysis solution, 4×10^5 leukocytes were used for the antigenemia test according to the manufacturer's instructions, while the rest was processed for PCR amplification as follows: The leukocytes were washed once in PBS and resuspended in 2 ml TNE for DNA extraction. DNA was isolated by phenol extraction and ethanol precipitation and resuspended in 50 μl TE (10 mM Tris pH 8.0, 0.1 mM EDTA).

Competitive PCR

HCMV-specific amplification was done by gB1 (5'gaggacaacgaaatcctgttgcgca 3') and gB2 (5'gtcgacgggtggagatactgctgagg 3') primers [9], the PCR cycle profile for HCMV was 95°C 30 seconds–62°C 1 minute–72°C 1 minute over 35 cycles. β -globin sequences were amplified by pCO3 (5'acacaactgtgttactagc 3') and pCO4 (5'caacttcacccacgttcacc 3') primers [12]. The PCR cycle profile for β -globin was 95°C 30 seconds–62°C 1 minute–72°C 1 minute 30 seconds over 35 cycles. All PCR amplifications were done at a MgCl_2 concentration of 1,5 mM unless otherwise indicated. PCR products were excised and purified from low melting point agarose (2%) with QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The purified amplicons were cloned into pGEM-T Easy PCR cloning vector (Promega Corporation, Madison, USA). Recombinant plasmids were transfected into *E. coli* strain XL-1 as described by Chung et al. [13]. Recombinant colonies were screened by PCR amplification as follows: A pinhead amount of a colony was transferred into the PCR reaction mixture. The initial denaturation was three minutes long to promote the release and amplification of the plasmid DNA from the bacterial cell. PCR positive colonies were grown in large scale and plasmids were isolated with Wizard Plus Midipreps DNA Purification System (Promega, Madison, USA). The pgB (HCMV)

plasmid was further modified inserting random *Mbo*I fragments of human fibroblast genomic DNA into the *Bgl*II site. For the quantitative competitive PCR, the competitor plasmids of extended lengths were added to the PCR reaction. The PCR products were subjected to an electrophoresis in 1.5% agarose gel and stained with ethidium-bromide. The density of the wild band was compared with the density of the mutant band with Gel Doc 2000 Gel Documentation system (Bio RAD Laboratories Hercules, C. USA) as follows: a calibration curve was set by plotting log (calibrating copy number) against $\log ([\text{band density of wild type amplicon}] / [\text{band density of wild type} + \text{band density of competing amplicon}])$.

Results

Cloning competitive mutant sequences

A 150 bp sequence of the glycoprotein B gene (gB) was amplified from human fibroblast cells infected with human cytomegalovirus (HCMV strain AD 169) and was cloned into pGEM T-Easy PCR cloning vector. Recombinant plasmid (pgB) was isolated in large scale from two recombinant colonies. The sensitivity limit of the PCR amplification was 10^3 copies using any of the two pgB isolates. To increase the insert size of the pgB plasmid, we ligated random *Mbo*I fragments of human fibroblast genomic DNA into the unique *Bgl*II restriction site of the gB amplicon. Approximately twenty recombinant colonies were PCR amplified with HCMV primers (Fig. 1). Two recombinant colonies with about 350 (pgBm1) and 400 (pgBm5) bp insert, respectively, were selected for large-scale plasmid preparation. The sensitivity limit of the PCR amplification of the mutant sequences of extended length was 10^3 copies.

A 110 bp sequence of the β -globin gene was amplified from human fibroblast cells and was cloned into pGEM T-Easy PCR cloning vector. Recombinant plasmid (pFB) was isolated in large scale from two recombinant colonies. The sensitivity limit of the PCR amplification was 10^3 copies using any of the two (pFB1 and pFB2) isolates. The β -globin mutant sequence was amplified from human fibroblast genome by increasing the MgCl_2 concentration. Aspecific bands appeared at a MgCl_2 concentration of 6mM (Fig.2 lane 1). The aspecific bands were excised and extracted from the agarose gel and reamplified in two consecutive PCR amplifications (Fig.2 lanes 2, 5, 6, 7). A 400 bp aspecific fragment apparently flanked by the β -globin primer sequences was cloned into pGEM T-Easy PCR cloning vector. Recombinant plasmids (pFBm1; pFBm2) were isolated in large scale from two recombinant colonies. The sensitivity limit of the PCR amplification was 10^3 copies using any of the two plasmids.

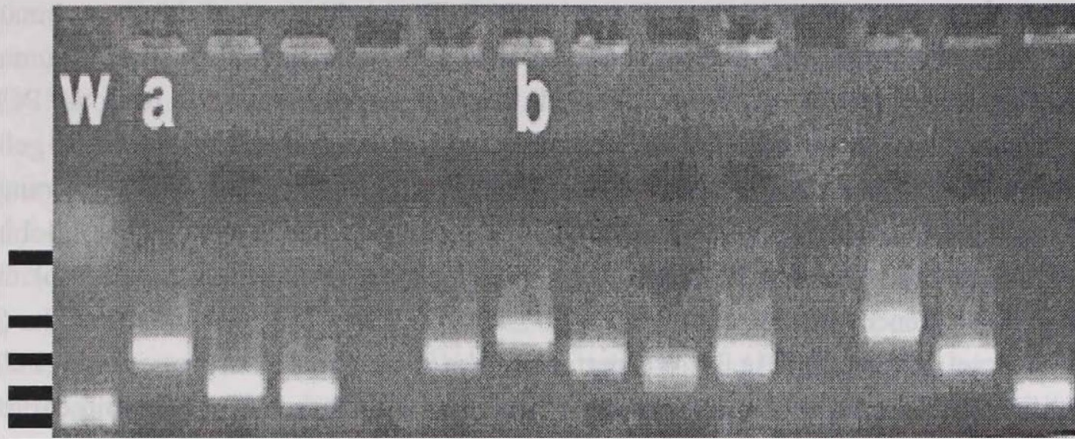


Fig. 1. Colony PCR after inserting random DNA fragments into the cloned 150 bp glycoprotein B sequence (pgB), (w) wild type pgB control (150 bp), (a, b) recombinant plasmids (pgBm1: 350 bp, pgBm5: 400 bp) used for competitive PCR. Marker bands are 500, 400, 300, 200 and 100 bp long

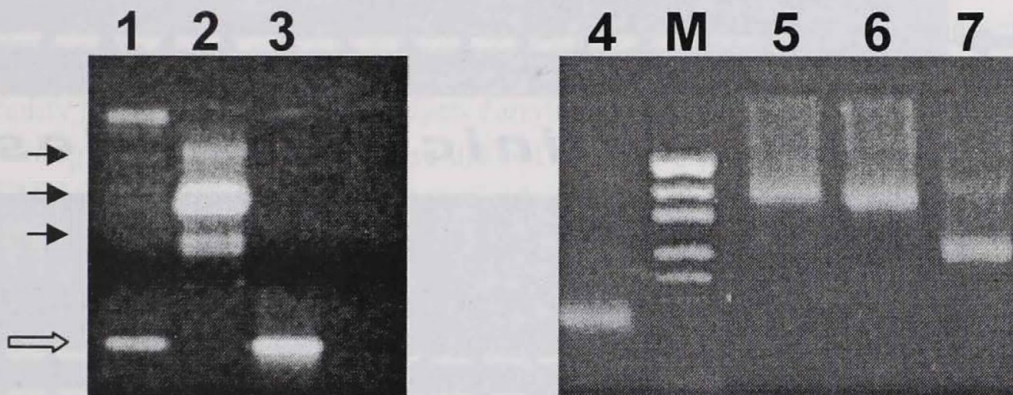


Fig. 2. Amplification of wild type and mutant β -globin sequences. White arrow indicates a 110 bp sequence of the β -globin gene amplified from human fibroblast cells. Black arrows indicate aspecific PCR products at 6 mM $MgCl_2$ concentration (lane 1). The aspecific PCR products are reamplified (lane 2). The 110 bp long sequence is reamplified (lane 3). Further reamplification of the wild type (110 bp) sequence (lane 4) and the aspecific bands (lanes 5–7). Marker bands are 500, 400, 330, 240, 190, 150, 110 and 70 bp long (M)

Competitive PCR

The quantitative PCR method was based on the competition of templates. The lengths of the natural genomic fragments flanked by the primer sequences were 150 bp and 110 bp for the human cytomegalovirus (HCMV) and for the β -globin, respectively. The cloned mutant sequences of extended lengths (pgBm1, pgBm5, pFBm1, pFBm2)

were used as competitors in the quantitative PCR. Serial dilutions of the recombinant wild type clones (pgB, pFB) were used to set the calibration curve (Fig. 3). For human cytomegalovirus (HCMV), the amount of the competitor necessary for one PCR amplification was optimized and 10^3 competitor copies were found to provide a good quantitative estimation of the wild type sequences in the range of 10^2 – 10^6 . This range was expected to cover the number of the HCMV genomes to be detected. For β -globin, 5×10^5 competitor copies were found to provide a good quantitative estimation of the wild type sequences in the range of 10^3 – 10^8 , covering the expected number of the β -globin target sequences (Fig.3). We performed the calibration in every series of PCR amplification. The HCMV copy number was related to the number of β -globin copies. β -globin band intensities were rather uniform indicating that the applied DNA isolation method was reproducible.

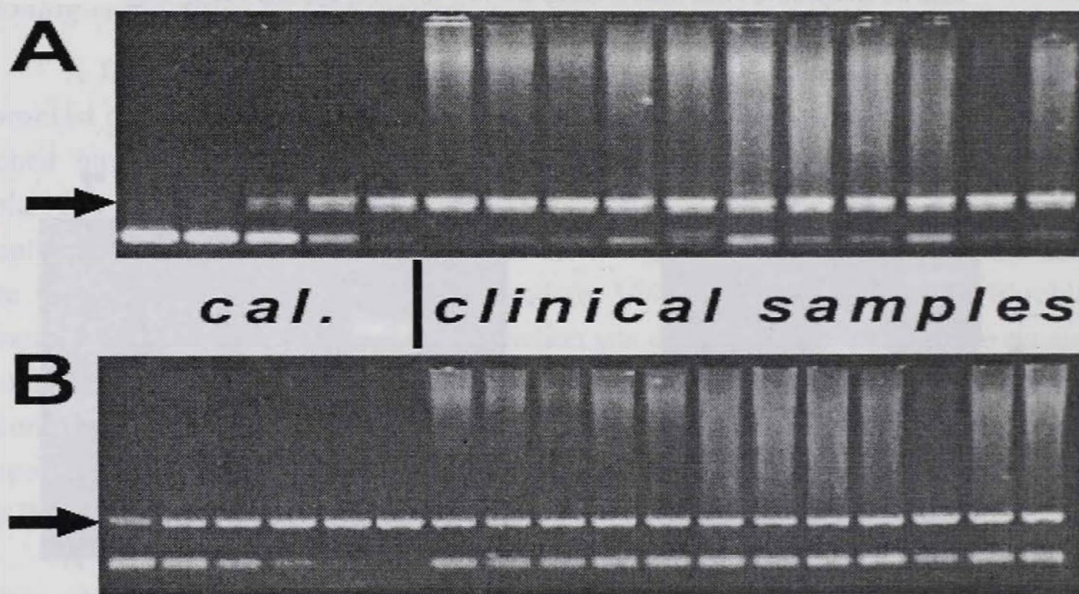


Fig. 3. Competitive PCR test of clinical samples. Arrows indicate the competitor bands. (A) HCMV amplification, 10^3 competitor target copies/reaction, (cal.) calibrating series: 10^6 – 10^2 copies/reaction. (B) β -globin amplification, 5×10^5 competitor target copies/reaction, (cal.) calibrating series: 10^8 – 10^3 copies/reaction

The system was tested on the blood samples of nine healthy blood-donors and seven renal transplant recipients (Fig. 3). In case of healthy blood donors (9 donors), the HCMV load ranged from 5 to 124 / 10^6 β -globin copy (median: 18 / 10^6). Four renal transplant patients who were negative by antigenemia test had 16–48 HCMV copies / 10^6 β -globin copies (median: 37) i.e. the HCMV load of the healthy blood

donors and the antigenemia negative renal transplant patients tended to be in the same range. Three renal transplant recipients positive in antigenemia test had indeed high HCMV load in the peripheral blood. Patient #1 with 30 antigenemia positive cells / 2×10^5 leukocytes had an HCMV load of 2.2×10^5 / 10^6 β -globin copies, patient #2 with more than 100 antigenemia positive cells / 2×10^5 leukocytes had an HCMV load of 1.5×10^4 / 10^6 β -globin copies and patient #3 with 45 antigenemia positive cells / 2×10^5 leukocytes had an HCMV load of 6.5×10^3 / 10^6 β -globin copies. It is of interest that the positive PCR result of the patient #2 was detected 20 days before his positive antigenemia test indicating that DNA-based detection of HCMV replication can detect the reactivation of the virus infection earlier than the routinely used antigenemia test.

Discussion

Human cytomegalovirus (HCMV) infection can cause acute life-threatening disease in organ transplant recipients. We have developed a competitive PCR method to detect HCMV reactivation in renal transplant recipients. To produce mutant sequences of extended lengths, we changed $MgCl_2$ concentration and the annealing temperature. For β -globin, the increase of $MgCl_2$ concentration made it possible to amplify longer aspecific PCR products carrying the primer sequences at the ends, while lowering the annealing temperature did not result in aspecific longer bands. With the HCMV gB primers, however, changing either the $MgCl_2$ concentration or the annealing temperature did not result in aspecific amplicons. The other attempt was to introduce an *HpaI* restriction site into the wild type gB sequence with PCR mutagenesis as described by Fox et al. [10]. Although this method worked well and provided reproducible measurements in competitive PCR, it required an extra restriction enzyme cleavage step after the PCR amplification, and the *HpaI* restriction fragments (75 bp) could be distinguished only in special agarose gels of high concentration (3–4%). The occurrence of a unique *BglII* site in the wild type gB sequence was optimal to increase the size of the cloned sequence by inserting additional DNA fragments, which were produced with the isoschizomer *MboI*. In contrast to the 6 bp recognition site of *BglII*, *MboI* recognizes only 4 base pairs, and thereby cleaves shorter random fragments. Eventually, we selected two mutant plasmids, where the insert was extended approximately by 200 bp and 250 bp, respectively.

There were marked differences in length between the competing and wild type sequences. Supposing that the target copies are amplified at a rate equal or very similar to each other, the longer PCR product will yield a larger amount and a stronger intensity i.e. the density ratio between the bands of different length does not apply to

the ratio between the competing and wild type sequence copies. Therefore calibration was done with a dilution series of cloned wild type target sequences, and this calibration was run in every PCR amplification in order to rule out any variation between the individual experiments.

We examined blood samples from healthy blood-donors and renal transplant recipients. According to our experiences, the amount of blood used to isolate DNA was critical. When only 200 µl blood was processed with commercially available DNA isolation kits, the PCR signals were inconsistent in the healthy blood donors [3]. Therefore leukocytes were isolated from 1.5 ml blood by lysing the red blood cells and then the DNA was phenol extracted from the leukocytes. Approximately one tenth of the isolated DNA was tested in a PCR reaction. This amount of DNA sample was found sufficient to amplify HCMV in a consistent manner. In conclusion, the quantitative measurement of HCMV load in the peripheral blood correlated well with the routine HCMV antigenemia test. The DNA-based test, however can detect earlier the reactivation of the HCMV infection.

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RECOMBINANT ADENOVIRUS VECTORS FOR GENE THERAPY AND CLINICAL TRIALS

(A REVIEW)*

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In the last decade adenovirus (AdV) vectors have emerged as promising technology in gene therapy. They have been used for genetic modification of a variety of somatic cells *in vitro* and *in vivo*. They have been widely used as gene delivery vectors in experiments both with curative and preventive purposes. AdV vectors have been used in the experimental and in some extent in the clinical gene therapy of a variety of cancers. The combination of recombinant AdV technology with chemotherapy (pro drug system) seems to be promising, too. AdV vectors offer several advantages over other vectors. Replication defective vectors can be produced in very high titers (10^{11} pfu/ml) thus allowing a substantially greater efficiency of direct gene transfer; they have the capacity to infect both replicating and nonreplicating (quiescent) cells from a variety of tissues and species. Several important limitations of adenovirus mediated gene transfer are also known, such as the relatively short-term (transient) expression of foreign genes, induction of the host humoral and cellular immune response to viral proteins and viral infected cells, which may substantially inhibit the effect of repeated treatment with AdV vectors, the limited cloning capacity and the lack of target cell specificity. However, the well-understood structure, molecular biology and host cell interactions of AdV-s offer some potential solutions to these limitations.

Keywords: adenovirus, adenovirus genome, recombinant adenovirus vectors, clinical trials

* This paper was written to commemorate to the fiftieth anniversary of the foundation of the Hungarian Society for Microbiology.

Characteristics of adenoviruses essential for vector construction

Adenoviruses have a linear double stranded DNA genome of about 35 kbp. The DNA is packaged in an icosahedral protein capsid, which is about 70–90 nm in diameter. The capsid is composed of 252 capsomers, 240 of which are hexons and 12 of which are pentons located at the 12 vertices of the capsid. A rod-like structure called fiber protrudes from each penton base [1]. Each DNA strand has inverted terminal repeats (ITR) of 100–140 bp necessary for viral replication. Their length is dependent on serotype. The genome is divided in early (E) and late (L) regions expressed before or after replication of the viral DNA, respectively (Fig. 1). There are six early regions: E1A, E1B, E2A, E2B, E3 and E4 that are transcribed from individual promoters (except E2). The two transcription units of E1 region (E1A and E1B) are the first part of the genome to be expressed following virus entry into a cell via endocytosis. In the first step of the entry the fiber binds to a specific receptor, then the penton base protein binds to integrins on the cell surface. This latter step promotes the internalization of the virion. Both E1A and E1B proteins are involved in the control of viral gene transcription. E1A proteins are required for virus replication, while the E1B proteins increase and decrease host mRNA transports and prevent apoptosis induced by E1A. It is worth mentioning, however, that the oncogenic AdV type 18 has been isolated from a urological tumor [3]. The presence of antibody to the early non-virion antigens of oncogenic AdV type 12 has been confirmed in more than 50% of the patients with urological tumors, in contrast to the 18% incidence in other urological patients with no tumors, and 4% incidence in patients suffering in some internal disease. Antibodies against the complete virions have been found in 15% and 2%, respectively in the same groups. These data seem to suggest that the genes of the early AdV proteins should be present and operate (expressing the early /onco/ proteins) in fairly high proportion of patients with tumors of urogenital tract, since induction of the non-virion antigens and antibodies against them could not be possible without their effect [4].

The genes of the E2A region encode the DNA binding protein and the E2B encode the DNA polymerase and the terminal protein. All of these proteins are directly implicated in the viral replication. The E3 region encodes proteins that interfere with the host immune response against virus infection. This region is not required for AdV replication [5]. Proteins encoded by the E4 region play a role in viral RNA transport and stability, viral DNA accumulation and transcriptional regulation of E2 and E4 [6].

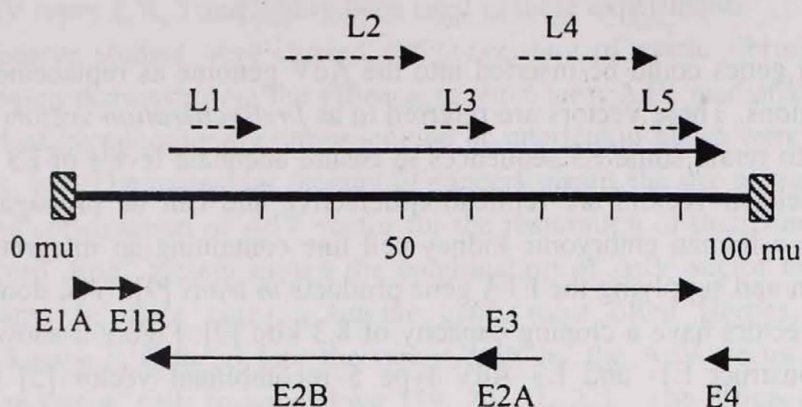


Fig. 1. Transcription map of the adenovirus genome showing the major transcription units. The thick horizontal line represents the 0–100 map units (mu) of the linear double stranded DNA. The genome is divided in six early (E) and five late (L) regions, respectively, expressed before or after the replication of the viral chromosome

Adenovirus vectorology

Since the early description of the development of AdV vectors for the expression of heterologous genes by Berkner in 1986 [7] several hundreds or even more different adenovirus vectors have been constructed to date and tens of thousands of scientific articles have been published about them. AdVs are now being increasingly investigated as potential mammalian expression vectors for gene therapy and for recombinant vaccines. Modifications of the vector backbone, the introduction of helper-dependent AdV vectors, the generation of hybrid AdV vectors, the targeted gene delivery by tropism-modified adenoviral vectors, the combination of these vectors led to the development of “Adenovirus vectorology” as a distinct research field of gene delivery techniques.

FIRST-GENERATION ADENOVIRUS VECTORS

Foreign genes could be inserted into the AdV genome as replacements for the E1 and E3 regions. These vectors are referred to as *first-generation vectors*. It may be advantageous to retain some E3 sequences to ensure adequate levels of E3 expression [8]. The E1-deleted vectors are replication-defective and can be propagated in 293 cells, which is a human embryonic kidney cell line containing an integrated copy of AdV E1 region and supplying the E1A gene products *in trans* [9]. The doubly deleted E1–E3 AdV vectors have a cloning capacity of 8.3 kbp [2]. Figure 2 shows a typical strategy to construct E1- and E3 AdV type 5 recombinant vector [5] by *in vivo* homologous recombination. Efficient procedures were developed to select recombinant viruses [10].

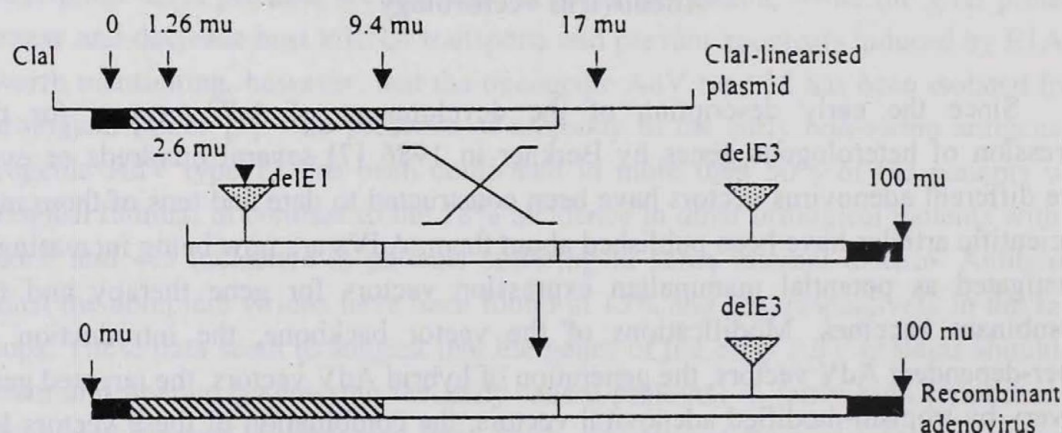


Fig. 2. Construction of an E1 replication defective adenovirus vector by homologous recombination. The transgene (▨) is inserted in a plasmid (—) between the 0–1.26 mu left end of the AdV containing the LTR and packaging sequences (■) and an AdV genome fragment from 9.4 to 17 mu (□) to permit *in vivo* homologous recombination. AdV vectors are generated after cotransfection of the ClaI-linearized chimerical plasmid and the ClaI-restricted AdV genome (□) into 293 cells. (delE1: E1-deletion, delE3: E3 deletion; *in vivo* recombination) [5]

First-generation AdV vectors have been used successfully for many short-term applications for genetic modification of a variety of somatic cells (lung, liver, muscle, blood vessels, brain) *in vitro* and *in vivo* [11]. In animal models short-term expression is sufficient for AdV vector based vaccines to protect against numerous pathogens such as Hepatitis B, HSV, HCMV, VSV, HIV, Parainfluenza virus type 3, Rotavirus,

Coronavirus, Malaria, Leishmania [2, 5, 12]. For the construction of recombinant vectors AdV types 2, 4, 5 and 7 have been used in these experiments.

Extensive studies were carried out concerning of cystic fibrosis [15] and of cancer research demonstrating the efficacy of short-term AdV mediated gene therapy. In the field of immunotherapy tumor antigen or interleukin genes were carried by the vectors [13, 14]. The molecular therapy of cancers means the use of tumor suppressor genes in the construction of AdV vector for the restoration of their functions [16, 17, 18]. The "pro drug" system means the combination of AdV vector technology with chemotherapy. In these cases a suicide gene, most often Herpes simplex virus thymidine kinase is induced into the tumor cells by the AdV vector, which makes sensitive the tumor, cells to ganciclovir [19, 20, 21, 22]. The efficacy of short-term AdV-vector expression was demonstrated also by blocking proliferation and by preventing the growth of new vessels to the tumor [23, 24].

Nevertheless there are at least four limitations outlined in the Introduction associated with the use of the first generation AdV-vectors. To overcome all the problems and obstacles it was necessary to construct other different types of AdV vectors and in addition to modulate the host immune response.

Second-generation vectors and the circumvention of the immune response

The most serious obstacles in the use of AdV vectors for long-term gene therapy is the induction of the host immune response and the relatively short transgene expression. The humoral and cellular immune response to viral proteins and viral infected cells substantially limits the level of gene transfer and eliminates AdV-infected cells especially in case of repeated treatment with the same AdV vector. In patients exposed to AdVs the pre-existing immunity to AdV-vectors could block even the effect of the first treatment, too [2, 25, 26]. Further crippling of the vector backbone was thought to be useful in the improvement of first generation vectors to decrease the host immune response and to increase the duration of transgene expression. Another risk of the first generation vectors also became known, namely that replication competent adenoviruses (RCA) may emerge by homologous recombination between identical sequences in the 293 chromosome and the viral genome, that may progressively outgrow the recombinant virus during propagation. To solve this latter problem novel A549-derived and other complementation cell lines was developed as well [34, 35]. For construction new AdV vectors the inactivation/deletion of E2A, E2B and E4 regions have been targeted in addition of deletion of the E1 region [36]. Generally these vectors

are referred too as *second-generation vectors*. Ablation of E2A or E2B in AdV vectors improved transgene persistence and decreased inflammatory response in mouse liver [37]. E4 region is a complex regulatory unit that encodes 7 polypeptides, subvert endogenous gene expression at different levels (transcriptional and post-transcriptional). E4 ORF3 and E4 ORF6 proteins especially show redundant activity. Several groups have studied the approach of crippling viral gene expression by deletion of the E4 region [2, 35, 36, 38]. Different vector systems have been developed to decrease or block the viral gene expression. With the deletion most of the E1 and E4 regions it is possible – and it proved to be advantageous – to retain some part of the E4 for the *in vitro* high level expression of transgene and for reduction of the level of viral proteins. The *in vivo* obtained results, however, are conflicting concerning the effect of E4 deletions on the persistence of transgene expression and on the immune response, therefore further studies are needed [2, 39, 40]. Another way to block the immune response could be the transient immunosuppression of the host with immunosuppressants concurrent with AdV vector injection, which permits vector readministration, and may increase the transgene expression [27, 28]. Agents that target specific cell types or interactions necessary for immune activation have been also studied. Inactivation of CD4 cells was shown to increase the duration of transgene expression. Cellular and humoral anti-AdV immune responses can be blocked by inhibition of the interaction between T cells and antigen-presenting cells. Antibody to CD40 ligands inhibits both types of immune responses and facilitates repeated administration of AdV vectors. Transient immunomodulation with anti-CD40 ligand antibody and CTLA4 Ig with concurrent AdV vector administration enhanced persistence and secondary gene transfer into mouse liver [2]. Circumvention of anti-AdV humoral immune defenses against repeated AdV vector administration by changing the adenovirus serotype (“sero-switch”) was also shown to be useful [29, 30]. However, the AdV types used sequentially should be well chosen because of the many intertype specific (IT) epitopes found in different numbers and combinations on the different AdV serotypes. The panels of monoclonal antibodies raised against 3 different AdV types recognized 18 different bi- and multilateral IT specific epitopes in addition to the genus and type specific epitopes on the 21 hexon types examined. Immune response of the host could be induced not only by the genus and the type specific epitopes, but also by the several IT specific ones. For the elimination of harmful immune response 31 AdV type pairs as well as small groups of types were determined showing the loosest antigenic relationship to each other. These pairs suggested to chose for preparation of second or multiple recombinant vectors. For instance, the most frequently and worldwide used vector for the experimental gene therapy is the HAdV type 5. For the second or multiple administration AdV vectors

could be used prepared from (i) bovine AdV type 3 which has no at all identical IT specific epitopes with type 5, although the number of the different epitopes on the two types is 15, (ii) HAdV type 35 which has 5 identical epitopes, but the two types contain 13 different epitopes, (iii) bovine AdV type 2 having 5 identical epitopes, and the two types contain 11 different epitopes, (iiii) HAdV type 12 having 6 identical epitopes with type 5 and the two types contain 11 different epitopes on their hexons [31, 32, 33].

Third-generation vectors helper-dependent ("gutless") vectors

Several groups reported the construction of AdV vectors with all or nearly all of the transcription units removed from the viral backbone except the ITRs and the packaging signal. Such vectors could be referred as *third-generation vectors*. In all these cases the viral proteins required for replication should be provided *in trans* by using a replicative transcomplementation approach [2, 36, 41]. For example, the LTU can be provided *in trans* within a helper adenovirus. This strategy extends the cloning capacity of the vector up to 37 kb and decreases the immune response to the minimal level possible. As these vectors are propagated in the presence of a helper virus (*helper-dependent vectors*) extensive purification is required to reduce the amount of contaminating helper virus of the production batch. Because of its smaller density the mini-adenovirus i.e. the helper-dependent adenovirus (HDAV) vector can be separated from the *wt* or first generation helper AdV by CsCl density gradient centrifugation. Construction of a helper-dependent mini-adenovirus is shown in Fig. 3 [36, 41]. The transcomplementation of the LTU is also possible *in cis* before its removal from the backbone of an AdV vector during viral amplification. Specific excision of large adenoviral helper sequences has been achieved with the loxP specific Cre recombinase from bacteriophage P1 resulting in a minivirus genome of about 9 kb that retained in addition to the transgene, a single loxP site and the E4 region of AdV genome [2, 36, 42]. Construction of a helper-dependent AdV vector directly from a first-generation vector is shown in Fig. 4. An alternative and more effective Cre-mediated system has also been developed in which the packaging element was flanked by loxP sites in a first-generation helper virus. Transfection of 293 Cre cells with HDAV vector followed by infection with the helper virus resulted in selective excision of the packaging element from the helper virus and preferential packaging of the HDAV vector [2, 43, 44]. After density gradient centrifugation the helper virus contamination decreased to as low as 0.01 % and *in vivo* administration of the vector

resulted in high expression levels for nearly a year. However, the maintenance of stability and the prolonged expression need appropriate structural design and production methodology to be implemented [2, 45, 46].

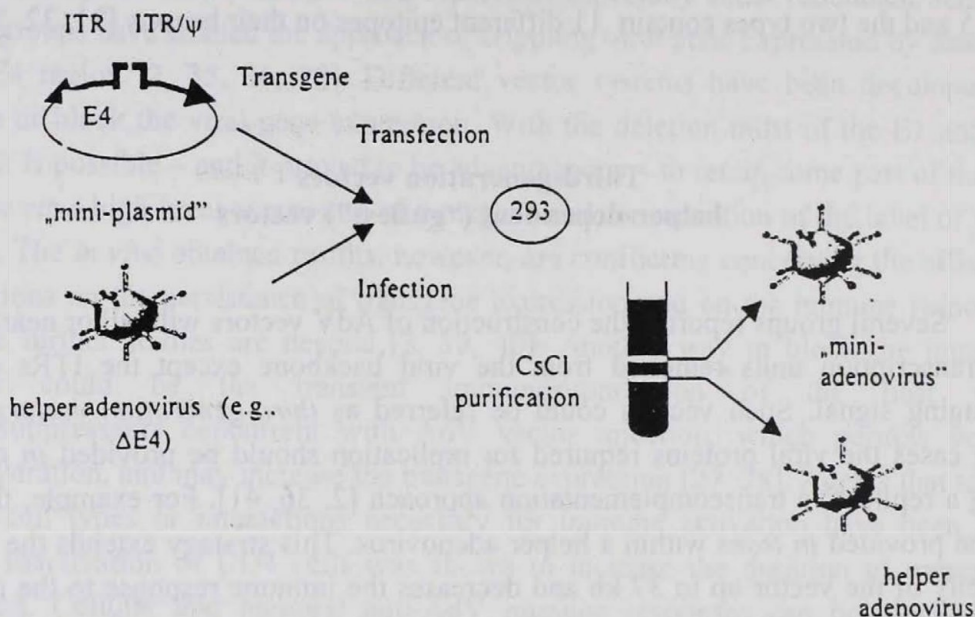


Fig. 3. Construction of helper-dependent "mini-adenovirus" with "mini-plasmid" carrying minimal AdV genome including ITRs, packaging signal and E4. The plasmid generates the mini-adenovirus in 293 cells with E4 defective helper AdV. The presence of E4 in the mini AdV and its absence in the helper virus ensure its propagation [36, 41]

Hybrid vectors, vector chimeras

Because of the relatively short-term expression of foreign genes associated with AdV vectors due to their non-integrating DNA, chimerical vector systems have been developed that combines the high efficiency *in vivo* gene delivery characteristics of AdV vectors with integrative capacities derived from retroviruses or from Adeno Associated Viruses (AAV) [47, 48]. In AdV-Retrovirus system this was accomplished by rendering AdV vector-infected target cells into retroviral producer cells by AdV-mediated delivery of retroviral packaging functions with one vector. By this way the locally elaborated retroviral vectors can infect neighboring parenchyma cells via an integrative vector as shown in Fig. 5 [47].

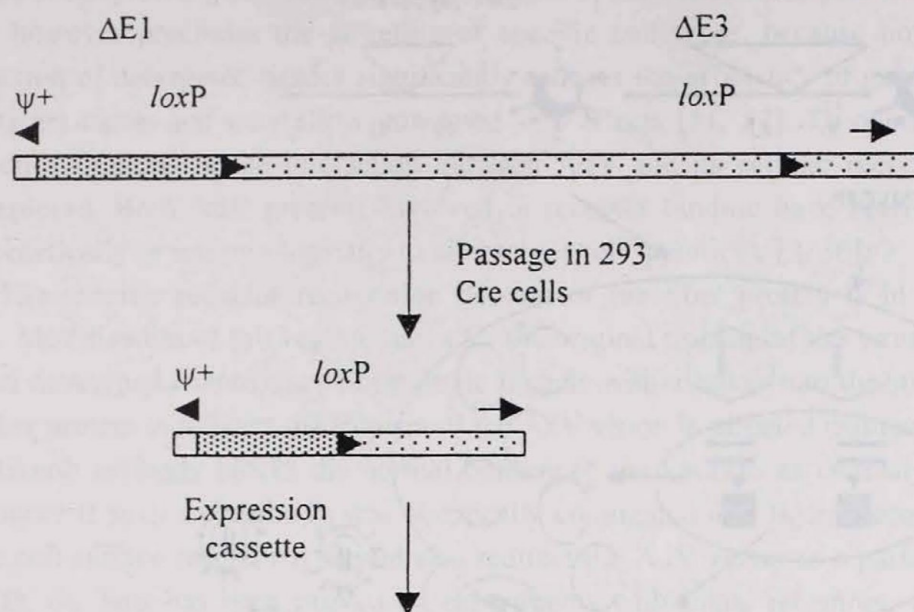


Fig. 4. Construction of helper-dependent AdV vector from a first-generation vector. A first-generation AdV vector was constructed with a transgene expression cassette (hatched box) upstream of a loxP Cre recombinase site (black arrowhead) in place of E1, and a second loxP site in place of E3. Amplification of this virus in Cre expressing derivative of 293 cells resulted in the excision of all viral sequences between E1 and E3 and generated the mini HDAdV vector shown, which was replicated and packaged by transcomplementation directly from the first-generation vector [2, 36]

In AdV-AAV hybrid – one of the first hybrid vector – a first-generation AdV vector contained *lacZ* flanked by AAV ITRs in the E1 region and when a plasmid carrying AAV *rep* gene was conjugated to the first hybrid in infected cells efficient *lacZ* expression was shown. The AAV *rep* gene is necessary for AAV replication and rescues from the AdV genome [49]. In an other AdV-AAV system, site-specific transgene integration (in human chromosome 19) was observed in cells coinfecting with one HDAdV vector encoding AAV *rep* gene, and another HDAdV vector encoding a transgene flanked by AAV ITRs [48, 50].

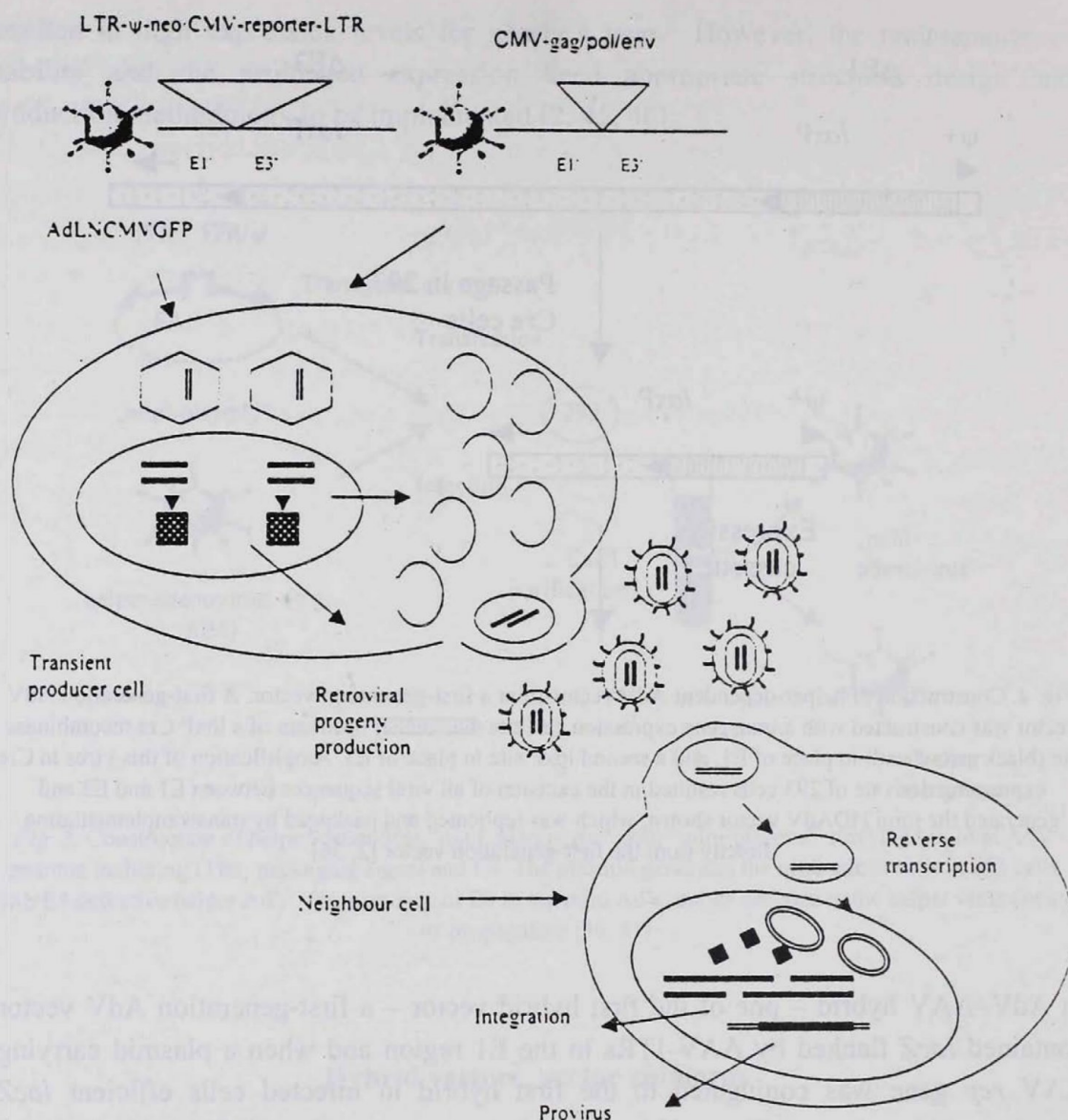


Fig. 5. Local generation of retroviral vectors at target organ site by AdV-retrovirus hybrid vectors. AdV vectors encoding retroviral vector and packaging functions accomplished *in vivo* gene transfer to target cells, rendering them retroviral producer cells. The locally produced retrovirus virions can thus directly infect neighboring cells [47]

Genetic engineering of the adenovirus capsid targeted vectors

There are two AdV coat proteins, which interact with distinct cellular receptors during the infection process. The fiber protein mediates the viral attachment to a cellular receptor. Following the attachment to cells, the penton base binds to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors mediating AdV internalization into the host cells by receptor-

mediated endocytosis. The widespread distribution of the cellular receptor for the fiber protein however precludes the targeting of specific cell types, because non-specific transduction of untargeted tissues significantly reduces the efficiency of gene delivery to the target tissue and may allow unwanted side effects [51, 52]. To overcome this limitation alterations in the interaction between AdV and its cellular receptors have been explored. Both AdV proteins involved in receptor binding have been modified either genetically or immunologically to alter target cell specificity [2, 36].

The specific receptor recognition domain of the fiber protein is in the knob portion. Modification of this region may alter the original tropism of the virus. Method has been developed to introduce physiologic ligands with a linker into the structure of AdV fiber protein to redirect the tropism of the AdV virion in targeted manner [51, 53]. The antiknob antibody blocks the normal binding of the knob to its cellular receptor. Furthermore if such an antibody was chemically conjugated to a ligand recognizing a specific cell surface receptor it should also redirect the AdV vector to a particular cell type (Fig. 6). This has been proved by experiments with folate receptors, which are over expressed on the surface of several malignant cell lines (ovarian, lung and breast cancers). Folate conjugated Fab fragment of an antiknob monoclonal antibody complexed with an AdV vector is able to redirect AdV infection to target cells [54, 55]. Coupling of Fab fragment to fibroblast growth factor also successfully redirect AdV vector to cells expressing ligand receptor [56]. The genetically modified fiber at the carboxyl terminus or H1 loop can also alter the tropism of AdV vectors [51, 57].

Cells lacking the α_v integrins on their surface (nasal epithelial cells, muscle cells, monocytes, lymphocytes) are mainly resistant to AdV infections. If the wild type AdV integrin specific RGD sequences are changed to target cell specific sequences the modified proteins of penton base recognize the target cells' integrins and promote their infectability [58, 59]. In order to achieve AdV infection of cells without the fiber receptor (endothelial and smooth muscle cells) an AdV vector has been constructed (Fig. 7) in which the recognition sequence – the FLAG peptide epitope, DYKDDDDK – for a specific antibody is incorporated into the receptor-binding domain in the penton base. Complexing of this AdV vector with a bispecific antibody, comprising an antibody to the FLAG recognition sequence and an antibody to α_v integrins resulted in the targeted infection of cells lacking the AdV fiber receptor [54, 58, 59, 60, 61].

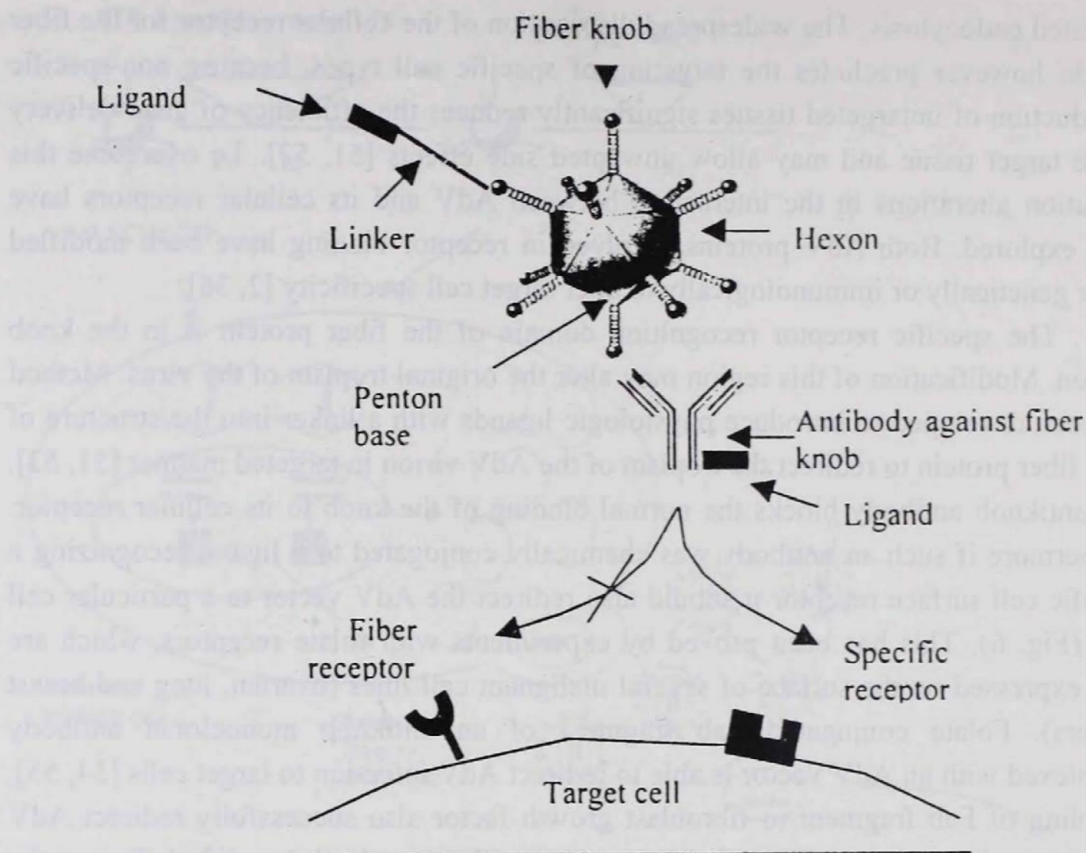


Fig. 6. Redirection of the tropism of adenovirus from its fiber receptor to the specific receptor of the target cell. This is possible with the introduction of a ligand with a linker into the fiber knob (upper left part), which specifically links to the surface receptor of the target cell. An antiknob antibody which blocks the binding of the virus to the fiber receptor and a ligand conjugated to the antibody recognizing the target cell receptor also redirect the adenovirus vector to a particular cell type (lower right part)

Adenovirus vectors in clinical trials

In the last twenty years genes of many disorders have been identified and diagnostic tests were developed. Hopes for gene transfer led to nearly four hundred gene-therapy clinical trials (for cystic fibrosis, immune deficiencies, cardiovascular diseases, cancer treatment, etc.). Preclinical *in vitro* and *in vivo* studies led to improvement of the AdV vectors used for transfer, but generally the effects obtained did not yet meet expectations. In the majority of the cases, either the gene correction was not enough to

reverse the clinical abnormalities, or it was limited by rapid extinction of transgene expression [62, 63].

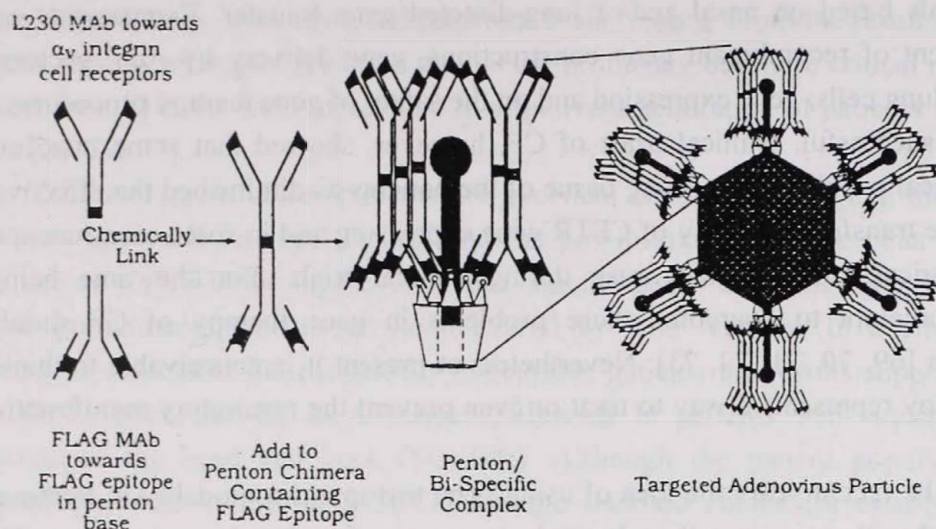


Fig. 7. Targeting adenovirus with bispecific antibodies to α_v integrins of host cells. Monoclonal antibody directed against α_v integrins is linked to another monoclonal antibody directed against the FLAG peptide epitope. Bispecific antibody is then added to AdV vector containing the FLAG epitopes incorporated into the penton base [59]

Lately, these trials have been extended to the treatment of AIDS and to cardiovascular diseases. Preclinical studies are in progress to evaluate the possibility of increasing the number of clinical trials for cardiopathies, and of beginning new gene therapy programs for autoimmune diseases, neurological illnesses, allergies, regeneration of tissues, to implement procedures of allogenic tissues or cell transplantation, and for the development of innovative vaccine design known as genetic immunization [63]. In the following part of this paper the use and exploitation of different recombinant AdV vectors in clinical trials conducted worldwide for the treatment of cancer, monogenic disorders and other diseases are shown on the base of several selected papers without any demand of completeness.

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). Mutations in the CFTR gene cause a loss of function of CFTR chloride channel and as a consequence the CF as a disease in electrolyte transport will be developed. There are a variety of clinical manifestations of CF, but the lung disease is the major cause of morbidity and despite of current standard therapy the median survival rate is below 30 years. CF was subsequently considered to be a candidate for gene therapy when the gene responsible for the disease was isolated and the research focused on the *in vivo*

approaches for gene transfer that could be delivered into the airway via aerosols. Soon after one of the first demonstration of complementation of CF cells using recombinant AdV vectors [64] clinical trials have been initiated [15, 65, 66, 67, 68] using a variety of protocols based on nasal and/or lung-directed gene transfer. Experiments on the development of recombinant gene constructions, gene delivery by AdV vectors into epithelial lung cells, gene expression and on the safety of gene therapy procedures were relatively successful. Clinical trials of CF, however, showed that some unaccounted physiological peculiarities of lung tissue of the patients as diminished the effectiveness of the gene transfer, longevity of CFTR gene expression and in some cases unexpected immunological complications arise during clinical trials. For the time being an intensive attempt to overcome these problems in gene therapy of CF should be undertaken [69, 70, 71, 72, 73]. Nevertheless at present it is conceivable to think that gene therapy represents a way to treat or even prevent the respiratory manifestation of CF.

In the recent years the idea of using gene therapy as a modality in treatment of diseases other than genetically inherited monogenic disorders has taken root. This is particularly obvious in the field of *oncology*. The vast majority of current gene therapy trials are for treatment of *cancer patients*, due to the recognition of gene alterations in cancer and the critical need for improvement of cancer therapy. Clinical trials include strategies that involve immunotherapy, induction of drug sensitivity in tumor cells or resistance to chemotherapy of critical host tissues and compensation for tumor suppressor loss or ablation of oncogenes. AdV vectors have been targeted to tumor-specific and tissue-specific antigens, such as epithelial growth factor receptor, c-kit receptor, and folate receptor. Targeted gene expression has been analyzed using tissue-specific promoters such as breast-, prostate- and melanoma-specific promoters, and disease-specific promoters such as carcinoembryonic antigen, HER-2/neu, Myc-Max response elements. Expression could be regulated externally by the use of radiation-induced promoters or tetracycline-responsive elements. Gene expression could be targeted at specific conditions, such as glucose deprivation and hypoxia [74, 75]. Another paradigm involves dendritic cells, potent antigen-presenting cells that play a critical role in the initiation of antitumor immune responses [76]. Using AdV vector transferring a gene encoding a specific tumor antigen this approach could be relevant in treating micrometastases present at the time of primary detection of many malignancies.

Brain tumors theoretically could be treated by targeting their fundamental molecular defects. During the malignant progression of astrocytic tumors several tumor suppressor genes are inactivated, and numerous growth factors and oncogenes are over expressed progressively. The p16/Rb/E2F pathway is the frequent target of genetic alterations in gliomas. Herpes simplex virus thymidine kinase (HSV tk) gene therapy

using AdV/tk vector combined with ganciclovir (GCV) medication seems to be a potential method for the treatment of malignant glioma, glioblastoma multiforme, gliosarcoma, anaplastic astrocytoma as it was shown in clinical trials involved 7 and 13 patients, respectively, with advanced recurrent brain tumors. From the results it is clear that gene therapy strategies for brain tumors are promising but more critical research is required, mainly in the field of vectors, to effective amelioration of patients with brain tumors [21, 22, 77].

Head and neck cancer patients are excellent candidates for gene therapy that offers new approach for local control and the possibility to enhance other treatment modalities as well. Tumor suppressor genes, suicide genes and genes whose products enhance immunocompetence can be delivered by AdV vectors. In a clinical trial involving 15 resectable but historically noncurable patients the results support the use of AdV-p53 gene transfer as a surgical adjuvant in patients with squamous cell carcinoma of the head and neck (SCCHN). Although the patient population with advanced or recurrent incurable SCCHN is the standard choice for establishing the safety of novel therapies the greatest chance of eventual success with currently available gene therapy strategies will most likely be in the patients with less advanced stages of the disease. Gene therapy in conjunction with the development of molecular diagnostics may provide the means for preventing the malignant progression of premalignant head and neck lesions upon early diagnosis [78, 79, 80]. A novel intervention strategy for SCCHN (and other tumors lacking p53) could be the use of AdV ONYX-015, which is an adenovirus with E1B 55 kDa gene deleted, engineered to selectively replicate in and to lyse p53-deficient cancer cells while sparing normal cells. In a phase II trial of a combination of intratumoral ONYX-015 injection (Adenovirus therapy) with cisplatin and 5-fluorouracil in patients with SCCHN by six months none of the responding tumors had progressed, whereas all non-injected tumors treated with chemotherapy alone had progressed [81].

Metastatic breast cancer and melanoma patients have been involved in a phase I trial of direct injection of an E1 and E3 deleted AdV vector encoding interleukin-2 into subcutaneous deposits of melanoma or breast cancer. 23 patients were injected at seven dose levels. The trial confirmed the safety of use of AdV vectors and demonstrated successful transgene expression even in the face of pre-existing immunity to AdV [14]. A HSV tk expressing AdV vector in combination with escalating doses of ganciclovir have been also used in a phase I study in patients with cutaneous metastatic malignant melanoma [82].

Non-small-cell lung cancer (NSCLC) is linked with p53 mutations. The p53 protein is a transcription factor that triggers cell cycle arrest and apoptosis in response to certain cellular stress and DNA damage. About 50 per cent of human tumors carry

p53 mutation; therefore attempts at p53 replacement are logical approaches to therapy in these diseases. Clinical trials of 15 and 28 NSCLC patients showed that the use of intratumoral injection of wild-type p53 complementary DNA (AdV-p53) is safe, well tolerated, feasible and biologically effective, results in transgene expression and seems to mediate antitumor activity [83, 84]. Another phase I and phase II trial demonstrated that P21 gene expression appears to be an indicator for the activity of AdV-p53 gene therapy [85]. Complementation of the gene therapy with simultaneous cisplatin/vinoreblin treatment was also examined in phase I and II trial studies including 21 patients with advanced NSCLC [86]. Cellular and humoral immune responses induced by AdV vectors were also studied in lung cancer patients [87, 88].

Malignant mesothelioma (MM) is a fatal neoplasm refractory to all forms of standard anticancer therapy. In a phase I dose-escalation clinical trial including 21 MM patients AdV mediated HSV tk/ganciclovir gene therapy was conducted. The study demonstrated that intrapleural administration of the vector was well tolerated and resulted in detectable gene transfer when delivered at high doses [89]. In an other phase I clinical trial including also 21 MM patients it was demonstrated that replication-defective AdV-tk vector administered intraperitoneally induced significant humoral and cellular immune response that induced no obvious adverse clinical sequel [90].

Chronic lymphocytic leukemia (CLL) cells can be made to express CD40-ligand (CD154) by transduction with a replication-defective AdV vector. *In vitro* transduced and bystander leukemia cells become highly effective antigen-presenting cells that can induce CLL specific autologous cytotoxic T lymphocytes. The immunological and clinical responses have been studied to infusion of autologous AdV-CD154-CLL cells in patients with CLL. Based on promising results, this approach may provide a novel gene therapy strategy for patients with CLL [91].

Colorectal cancer (CRC) is about the second commonest cause of cancer death. Death is commonly due to liver metastases and consequent progressive liver dysfunction. About 50% of CRC tumors have p53 alterations. Several gene therapy clinical trials in colon carcinoma are ongoing or in the approval process. Different gene therapy approaches can be employed such as tumor suppressor gene replacement with wild-type p53, enzyme/prodrug system, and immune gene therapy based on cytokine or tumor antigen expression to induce tumor immunity. Replication-deficient recombinant AdV vectors are predominantly used and can be administered by infusion via the hepatic artery for the regional gene therapy of malignant liver tumors. The patients studied generally have incurable metastatic CRC of the liver. Clinical trials provide data for design of future studies with respect to dose, form and frequency of administration, to evaluate the biological efficacy, efficiency and stability of gene transfer. Colon cancer gene therapy is likely to take new directions such as use as

adjuvant to radical surgery, rather than attempts to treat end-stage disease. Another direction might be either prophylactic gene-based immunization against a panel of well-characterized tumor antigens or the combination of gene therapy with conventional anticancer treatment such as radiotherapy and chemotherapy [16, 17].

Primary and secondary liver tumor patients with inresectable tumors were involved in phase I and phase II clinical studies with a recombinant mutant AdV with E1B 55 kDa deletion (d11520). The treatment was well tolerated when administered intratumorally, intraarterially or intravenously. The combination of d11520 and 5-fluorouracil infused into the hepatic artery was also well tolerated. However, in order to achieve better clinical response, further improvement in the vector design will be needed [16, 92].

Ovarian cancer (OC) gene therapy strategies employed various viral and nonviral vectors. Thus far AdV vector has been the most promising vector. Gene therapy approaches include different suicide gene strategies, replacement of wild type p53 and the use of an anti-erbB-2 antibody-encoding AdV vector. Novel advances in gene therapy approaches include refinement of vector targeting, the use of site-specific promoters and conditionally replicative AdV vectors. Phase I study of 14 patients with AdV HSV tk/GCV showed that the treatment was feasible in the context of human OC. In another suicide gene therapy trial including 10 patients acyclovir (ACV) or valacyclovir (VCV) were used as enzymatic substrate. It was concluded that replacing ACV by VCV would offer a cost-effective alternative. Another clinical trial of 10 patients with advanced epithelial ovarian cancer showed that i.p. delivery of AdV HSV tk/ACV and concomitant topotecan chemotherapy was well tolerated without lasting toxicity and side-effects were independent of dose of the AdV vector. In a phase I trial of 15 patients with recurrent ovarian cancer an anti-erbB-2 single chain antibody encoding AdV vector was used to treat erbB-2-overexpressing OC. The results demonstrated the feasibility of this strategy. In general it could be concluded that although several clinical trials have documented the relative safety of the used treatments, few significant clinical responses have been effected. Nevertheless gene therapy strategy does appear promising for the treatment of OC because advances in this field are occurring rapidly [19, 20, 93, 94, 95].

Bladder cancer (BC) frequently shows mutations in the p53 gene. AdV mediated p53 gene transfer is growth-inhibitory to BC cells. The AdV-p53 vector was administered intravesically to patients with BC in phase I trial, and provided basis for phase II and phase III trials [96].

Prostate cancer (PC) represents an accumulation of genetic mutations that causes prostate cells to lose the ability to control their growth, therefore gene therapy approach could be the replacement of tumor suppressor genes p53 and p16 with AdV

vectors containing wild type genes. Alteration in the p16 gene and its protein expression often occur in PC. Prostate tumors injected with AdV-p16 vector expressed p16 for up to 14 days and markedly suppressed tumors *in vivo* [97, 98]. A phase I clinical trial including 18 patients with PC was the first to demonstrate the safety of AdV/HSV-tk plus GCV gene therapy in human PC and the first to demonstrate the anticancer activity of gene therapy. In a further clinical trial 52 patients received intraprostatic injections of AdV/HSV-tk vectors followed by intravenous ganciclovir or oral valacyclovir injections. All toxic events after multiple or repeated injections were mild and resolved completely once the therapy course was terminated. It was concluded that direct injection into the prostate of AdV/HSV-tk gene followed by i.v. GCV is safe even in repeated cycles [99, 100].

Cardiovascular disease is the leading cause of death in the western part of the world and gene therapy approaches to several cardiovascular disorders including restenosis after angioplasty, therapeutic neovascularization, and bypass graft restenosis have been proposed. Phase I clinical trials have been reported, but convincing efficacy data in humans do not yet exist [101]. A six-month assessment of a phase I trial of angiogenic gene therapy for the treatment of *Coronary artery disease* was also reported. In this trial, 21 patients were given direct myocardial injection of AdV vector expressing the human vascular endothelial growth factor (VEGF) 121 cDNA to induce therapeutic angiogenesis. In 15 patients the vector was used as an adjunct to conventional coronary artery bypass grafting and in 6 patients as sole therapy via minithoracotomy. This study suggested that these methods appear to be well tolerated and initiation of phase II evaluation of therapy appears warranted [102]. It is worth mentioning that in a phase I trial including 30 patients with inoperable coronary artery disease direct myocardial transfer of naked DNA-encoding VEGF165 was used with promising success [103].

Wound healing is a particularly attractive approach for gene therapy. AdV-mediated gene transfer in wound healing is a relatively new application of this vector, but it seems to be a useful tool in the study of the role of specific cytokines in normal and impaired wound healing. Phase I trials are in progress with the AdV vector carrying platelet-derived growth factor A or B (PDGF) to study the local and systemic toxicity and the feasibility of using the maximum tolerated dose in chronic venous leg ulcers and chronic nonhealing diabetic foot ulcers [104, 105, 106].

The first gene therapy death

On 17 September 1999 an 18 years old boy with an inherited enzyme deficiency died four days after receiving AdV based gene therapy for treatment of partial ornithine transcarbamylase (OTC) deficiency. He was the first patient in a gene therapy trial to die of the therapy itself. He received a massive dose of vector – 38 trillion virus particles, the highest dose in this 18-patient trial – to try to get enough functioning OTC genes into his liver via hepatic blood vessel. The vector invaded not just the intended target (the liver), but also many other organs. The patient developed acute respiratory distress syndrome and died two days later of multiple organ failure due to anoxia. In spite of severe investigation, it remained a mystery why the patient died in consequence of the therapy. However, a list was presented of problems that need to be addressed to improve AdV safety. Clinicians have been urged to adopt a common index for dosing patients and a standard measure of virus particle concentration, database should be assembled on vectors and their effects, clinicians should be screen patients more carefully and researchers should collect better data on the fate of vectors in the body. The Recombinant DNA Advisory Committee (RAC) called for measuring transgene expression in cells and tissues, better assessment of immune status before and after dosing and studies of vector biodistribution [107, 108].

Conclusion and perspectives

The potential therapeutic application of gene transfer technology seems to be enormous. Many of the studies in inherited diseases and in cancer gene therapy clinical trials have provided information of critical importance for the design of efficient clinical protocols. Clinical trials have been extended to the treatment of many diseases. There are about thirty currently active gene therapy protocols for the treatment only of HIV-1 infection in the USA. These programs aim to confer protective immunity against HIV-1 transmission to individuals who are in risk of infection, to develop preventive or therapeutic vaccines for patients with AIDS and other infectious diseases. There is a proposal to begin in utero gene therapy clinical trials for the treatment of inherited genetic disorders. Gene therapy represents one of the most important developments in oncology, however, before this can be realized as standard treatment the technical problems of gene delivery and safety must be overcome. The early generation vectors are now likely to be phased out for most diseases. Researchers could turn to “gutless” vectors, which cannot reproduce, give much greater gene expression and are far less

inflammatory. Despite the latest significant achievements reported in vector design it is not possible to predict yet to what extent and when gene therapy will be effective.

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RELATIVE DEFICIENCY IN CpG DINUCLEOTIDES IS A WIDESPREAD BUT NOT UNIQUE FEATURE OF *GAMMAHERPESVIRINAE* GENOMES*

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Since methylcytosine is relatively unstable, a deficiency of CpG dinucleotides and accumulation of mutations that manifest as TpG (and its complement CpA) is a diagnostic feature of higher eukaryotic DNA sequences subjected to methylation by DNA (cytosine-5) methyltransferases. Latent viral genomes may also be affected by DNA methylation in their host cells. We calculated, therefore, frequencies of dinucleotides in 20 completely sequenced herpesvirus genomes. We found a relative deficiency of CpG dinucleotides and a surplus of TpG + CpA dinucleotides in all lymphotropic gammaherpesvirus genomes except for two strains of rhesus rhadinovirus. DNAs of two strains of human herpesvirus 7, a betaherpesvirus targeting helper T cells, and equine herpesvirus 4, an alphaherpesvirus residing in the lymphoreticular system, also had a moderate CpG deficiency and TpG + CpA surplus. In contrast, most members of *Alpha-*, and *Betaherpesvirinae* subfamilies contained a relative surplus of CpG dinucleotides in their DNAs. Our data are consistent with the idea that methylated latent genomes are involved, after reactivation and productive replication, in the natural transmission cycle of most members of *Gammaherpesvirinae* and certain lymphotropic members of *Alpha-* and *Betaherpesvirinae*.

Keywords: DNA methylation, CpG deficiency, herpesvirus genomes, Epstein-Barr virus

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Herpesviruses are widely distributed in nature. Their double-stranded DNA genomes are heterogeneous in size, base composition and organization [1]. There are around 40 conserved genes (the "core" set), which encode proteins involved in DNA replication, processing and packaging, capsid assembly and structure, cell entry and exit. Some of the genes for enzymes of nucleotide metabolism and repair also seem to be universal and belong to the "core" set. Less conserved ("non-core") genes encode control or modulator proteins, virion tegument and surface components. Highly divergent genes expressed during latency and/or associated with malignant cell transformation also belong to the "non-core" gene complement [1, 2]. All of the herpesvirus genomes are larger than 100 kbp. The viral DNA is transcribed, replicated and packaged into icosahedral nucleocapsids during productive (lytic, cytotoxic) replication. Productive cycles of virus growth are usually followed by establishment of viral latency and the site of latent infection is, in general, an important characteristic of herpesvirus subfamilies: *Alphaherpesvirinae* are neurotropic, *Gammapherpesvirinae* reside in lymphoid cells while the site(s) of residence for *Betaherpesvirinae* (monocytes and their bone marrow progenitors, endothelial cells) is still a subject of intense study. There are, however, exceptions since the equine herpesviruses 1 and 4 are classified as alphaherpesviruses on the basis of their genome structure and biological behavior but become latent in the lymphoreticular system [3]. In addition, the DNA sequence of human herpesvirus 6, a T lymphotropic virus [4], is most closely related to *Betaherpesvirinae* genomes [5]. Another betaherpesvirus, human herpesvirus 7, also targets helper T cells [6]. Based on dinucleotide frequency analysis performed on complete nucleotide sequence of a single lymphotropic herpesvirus (Epstein-Barr virus, EBV) and partial sequences of several other herpesviruses representing all three subfamilies, Honess et al. suggested that latent genomes of *Gammapherpesvirinae* are regularly methylated in their natural host cells and are or have been precursors to infectious progeny viruses [7]. They speculated that deficiency in CpG dinucleotides and excess in TpG and CpA dinucleotides in *Gammapherpesvirinae* DNAs might be due to deamination of 5-methylcytosine (which is relatively instable) and mismatch repair. Methylation of cytosines within CpG dinucleotides by eukaryotic DNA methyltransferases leads to similar deviations from expected dinucleotide frequencies in higher eukaryotic DNA sequences: relative deficiency in CpG due to mutations that generate TpG (and its complement, CpA) [8]. Since recently the number of completely sequenced herpesvirus genomes increased significantly [1, 9], we studied whether relative CpG deficiency is a general and exclusive feature of lymphotropic gammaherpesvirus genomes.

Methods

DNA sequences. Herpesviruses whose genomes are completely sequenced are listed (with their EMBL/GeneBank Accession Numbers) in Table I.

Analyses of DNA and presentation of results. Frequencies of mononucleotides and dinucleotides were calculated using a computer program written to count occurrences of k-tuples (J. Jurka and P. Klonowski, unpublished). The observed frequency of a dinucleotide XpY (f_{XY_o}) was calculated by dividing the number of occurrence of the XpY dinucleotide by the number of all dinucleotide positions. The expected frequency of an XpY dinucleotide (f_{XY_e}) was estimated from the frequencies of the X and Y mononucleotides as follows: $f_{XY_e} = f_X \times f_Y$. Observed and expected CpG frequencies are presented in Table II which also shows deviations of the observed from the expected frequencies of CpG dinucleotides as surplus (if the number of observed dinucleotides is higher than the number of expected dinucleotides) or deficit (opposite case). The sum of TpG + CpA dinucleotides was also calculated (Table II). Since the length of herpesvirus genomes differs from each other, normalized deviations of the observed from the expected values per 100 kbp were also calculated. A single strand of the listed DNA sequences was analyzed in each case.

Results

Table II shows that there were more CpG dinucleotides than expected in all three sequenced human alphaherpesvirus genomes and in a bovine alphaherpesvirus genome. Regarding equine alphaherpesviruses, CpG frequency was close to the expected value in the DNA of equine herpesvirus 1 but there was a moderate CpG deficit in the genome of equine herpesvirus 4. Among betaherpesviruses the human cytomegalovirus and murine cytomegalovirus genomes as well as the human herpesvirus 6 genome contained more CpGs than expected on the basis of their mononucleotide composition. In contrast, there was a moderate CpG deficiency in the genomes of both human herpesvirus 7 strains sequenced so far. All of the gammaherpesvirus genomes were deficient in CpG dinucleotides with the exception of two rhesus rhadinovirus strains. All of the herpesvirus genomes with CpG deficiency had a surplus of TpG + CpA (Table II). Deficit in CpG dinucleotides of EHV4, HHV7, HHV8 and HVS genomes was compensated with excess occurrences of TpG and CpA. In contrast, TpG + CpA surplus did not balance CpG deficiency in EBV, EHV2 and AHV1 and MHV4 genomes. These data suggest that factors other than cytosine

methylation may also affect frequencies of these dinucleotides in the genomes of lymphotropic herpesviruses.

Discussion

Based on the dinucleotide frequency analysis of the EBV genome and partial DNA sequences from representatives of alpha-, beta- and gammaherpesvirus genomes Honess et al. suggested that latent genomes of the lymphotropic *Gammaherpesvirinae* are extensively methylated and are or have been regular precursors of infectious progeny viruses with CpG-deficient genomes [7]. It is well established that latent EBV genomes carried by lymphoma or carcinoma cells regularly undergo *de novo* methylation [10–14]. Recent studies indicate that certain regions of latent EBV genomes are methylated in resting (memory) B cells of healthy individuals as well: the regulatory region of a promoter (Cp, also called BCR2) for transcription of latent messages coding for the nuclear antigens EBNA 1–6 was found to be partially methylated [15, 16] while a region around Wp (an alternative promoter for EBNA transcripts) was highly methylated [16]. Fp, a promoter used during productive virus replication but inactive during latency was also hypermethylated. In contrast, sequences around a third latent promoter, Qp (where transcripts for EBNA 1, but not EBNA 2–6 are initiated in certain host cells) were invariably unmethylated [16]. Robertson and Ambinder and Paulson and Speck speculate that methylation of the EBV genome may ensure (by switching off the promoters for genes encoding potentially immunogenic latent viral proteins) that a proportion of infected cells survive cytotoxic T-cell immune surveillance [15, 16]. Our data show that with the exception of two rhesus rhadinovirus strains all of the completely sequenced gammaherpesvirus genomes are relatively CpG-deficient and contain an excess of TpG and CpA dinucleotides (Table II) like higher eukaryotic DNA sequences that have been subjected to methylation by DNA (cytosine-5) methyltransferases. This implies that the majority of latent gammaherpesvirus genomes are methylated in their natural host cells. Table II also shows, however, that deviation from expected CpG frequencies is not an exclusive characteristic of gammaherpesviruses: two strains of human herpesvirus 7 (HHV7, a betaherpesvirus targeting helper T cells) are also deficient in CpG dinucleotides and contain a surplus of TpG + CpA. Lymphotropism *per se* can't explain this observation since the related human herpesvirus 6 (HHV6, which also targets T helper cells) has a CpG surplus (Table II). Genomes of other betaherpesviruses (HCMV and MCMV, which are not known to reside in lymphoid cells) contain an excess of CpG dinucleotides similarly to the HHV6 genome. Certain alphaherpesviruses (equine herpesvirus 1 and 4)

unexpectedly don't establish latency in neurons but become latent in the lymphoreticular system [3]. It is interesting to note that all of the *Alphaherpesvirinae* genomes sequenced contained a CpG surplus with the exception of EHV1 (which had a minimal CpG deficit, i.e. the observed CpG frequency was found to be very close to the expected one) and EHV4, which had a moderate CpG deficit. The CpG deficiency of the EHV4 genome was comparable to that of HHV7 strain JI. Our data show that lymphotropism of herpesviruses is frequently but not necessarily associated with CpG deficiency in their genomes and imply that methylated latent genomes are involved, after reactivation and productive replication, in the natural transmission cycle of these viruses. There is, however an example when methylated, latent genomes apparently do not play a role in transmission of the epidemiologically significant form of a herpesvirus which targets, among others, lymphoid cells: as discussed by Honess et al. [7], episomal genomes of Marek's disease virus (MDV, an alphaherpesvirus associated with lymphomas in chicken) are methylated in *in vitro* growing T lymphoblastoid cell lines derived from chicken T lymphomas [17] but the available samples of MDV DNA sequences are not CpG deficient. Although the complete sequence of the MDV genome remains to be determined and it is unknown whether MDV genomes are methylated in the tumor cells *in vivo* as well, it seems that MDV is either transmitted by virions produced after reactivation of unmethylated latent MDV genomes or by virions produced during lytic infection of lymphoid or nonlymphoid cells [7]. Lack of CpG deficiency in two strains of rhesus rhadinovirus, a gammaherpesvirus and human herpesvirus 6, a betaherpesvirus targeting T cells, could be explained in a similar way. Overall methylation patterns and promoter usage of latent EBV genomes correlate with the phenotype of their host cells: they are highly methylated in Burkitt's lymphoma cells, which maintain the phenotype of germinal center B cells, but hypomethylated in *in vitro* transformed lymphoblastoid cell lines of activated B blast phenotype [10, reviewed in 18]. Based on this observation we suggest that the host cells of HHV6 (whose genome has a CpG surplus) and HHV7 (with a CpG depleted genome) are different subsets of CD4⁺ T cells, which vary in their capacity for *de novo* methylation of latent herpesvirus genomes.

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Table I

Completely sequenced herpesvirus genomes

Virus subfamily and species	Virus name abbreviation	GeneBank/EMBL Accession Number	Genome length (bp)
<i>Alphaherpesvirinae</i>			
Herpes simplex virus type 1	HSV1	X14112	152 261
Herpes simplex virus type 2 (strain HG52)	HSV2	Z86099	154 746
Varicella-zoster virus	VZV	X04370	124 884
Equine herpesvirus 1 (strain Ab4p)	EHV1	M86664	150 223
Equine herpesvirus 4 (strain NS80567)	EHV4	AF030027	145 597
Bovine herpesvirus 1	BHV1	AJ004801	135 301
<i>Betaherpesvirinae</i>			
Human cytomegalovirus (strain AD169)	HCMV	X17403	229 354
Human herpesvirus 6	HHV6	X83413	159 321
Human herpesvirus 7 (strain JI)	HHV7	U43400	144 861
Human herpesvirus 7 (strain RK)	HHV7	AF037218	153 080
Murine cytomegalovirus	MCMV	U68299	230 278
<i>Gammapherpesvirinae</i>			
Epstein-Barr virus	EBV	V01555	172 281
Human herpesvirus 8	HHV8, KSHV [#]	U75698	137 508
Rhesus rhadinovirus (strain 17577)*	RRV	AF083501	133 719
Rhesus rhadinovirus (strain 26-95)**	RRV	AF210726	130 733
Murine herpesvirus 68 (strain WUMS)	MHV4	U97553	119 450
Herpesvirus saimiri	HVS	X64346	112 930
Equine herpesvirus 2	EHV2	U20824	184 427
Alcelaphine herpesvirus 1	AHV1	AF005370	130 608
Channel catfish virus (strain auburn 1)***	CCV	M75136	134 226

[#] Kaposi's sarcoma associated herpesvirus* Also called *Macaca mulatta* rhadinovirus, Rhesus macaque rhadinovirus and *cercopithecine* HV 17

** Also called Rhesus monkey rhadinovirus

*** Unclassified; also called ictalurid herpesvirus 1

Table II

CpG dinucleotide frequency analysis of herpesvirus genomes

Virus species	CpG frequency (%)		CpG surplus or deficit	CpG surplus or deficit per 10 ⁵ bp	TpG+CpA surplus or deficit	TpG+CpA surplus or deficit per 10 ⁵ bp
	Observed	Expected				
<i>Alphaherpesvirinae</i>						
Herpes simplex virus type 1	11.77	11.66	+170	+112	-244	-74
Herpes simplex virus type 2	13.10	12.38	+1111	+718	-808	-522
Varicella-zoster virus	6.01	5.29	+898	+719	+74	+59
Equine herpesvirus 1	7.99	8.02	-59	-39	+242	+161
Equine herpesvirus 4	5.95	6.36	-609	-418	+1246	+856
Bovine herpesvirus 1	15.60	13.11	+3364	+2486	-383	-283
<i>Betaherpesvirinae</i>						
Human cytomegalovirus	9.25	8.17	+3535	+1541	+1283	+559
Human herpesvirus 6	5.10	4.50	+956	+600	+329	+206
Human herpesvirus 7	2.50	3.12	-903	-623	+2539	+1753
Human herpesvirus 7	2.37	3.26	-1354	-885	+1577	+1030
Murine cytomegalovirus	10.55	8.62	+4429	+1923	-1860	-808
<i>Gammaherpesvirinae</i>						
Epstein-Barr virus	5.98	8.98	-6221	-3611	+2639	+1532
Human herpesvirus 8	5.78	7.15	-1877	-1365	+2378	+1729
Rhesus rhadinovirus*	7.51	6.88	+845	+632	+125	+93
Rhesus rhadinovirus**	7.47	6.80	+885	+677	+149	+114
Murine herpesvirus 68	2.38	5.57	-3813	-3192	+3649	+3055
Herpesvirus saimiri	0.98	2.96	-2237	-1981	+2923	+2588
Equine herpesvirus 2	5.19	8.26	-5666	-3072	+2037	+1104
Alcelaphine herpesvirus 1	2.21	5.31	-4057	-3106	+3189	+2442
<i>Unclassified</i>						
Channel catfish virus	8.81	7.90	+1214	+904	-330	-246

*Strain 17577

**Strain 26-95

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RESISTANCE TO β -LACTAMS AND GLYCOPEPTIDES IN STAPHYLOCOCCI AND STREPTOCOCCI

(A REVIEW)*

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Molecular mechanisms of the action of β -lactam and glycopeptide antibiotics, as well as genetic background and phenotypical features of the resistance of staphylococci, streptococci and enterococci to these antibiotics are reviewed. Furthermore, susceptibility patterns concerning β -lactam and glycopeptide drugs of staphylococcal, streptococcal, as well as enterococcal strains isolated from clinical specimens at the Semmelweis University of Medicine, Budapest, Hungary between January 1997 and December 2000 are also presented.

Keywords: β -lactam resistance, glycopeptide resistance, staphylococci, enterococci, streptococci

Introduction

Staphylococci, enterococci and streptococci are the most frequently isolated bacteria from extraintestinal clinical specimens. *Staphylococcus aureus* can cause both local, disseminated, disseminated generalized infections such as furuncle, abscess or postoperative wound infections and bacteraemia, endocarditis, meningitis, sepsis and osteomyelitis. Exfoliative toxin producing strains can also cause scalded skin syndromes and toxic shock syndrome toxin-1 producing ones can induce toxic shock syndromes, too. Coagulase-negative staphylococci (CNS) and enterococci are common causes of any – mainly catheter associated-nosocomial infections in premature infants

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and the elderly, as well as immunocompromised patients. Enterococci are among the most frequent microbes of nosocomial infections, and are selected by therapy with cephalosporins and other antibiotics to which they are naturally resistant. In patients, the most common sites of infection are the urinary tract, wounds, biliary tract, and blood. They may cause meningitis and sepsis in neonates, and endocarditis in adults. *Streptococcus pneumoniae* is one of the leading agents causing community-acquired bacterial pneumonia, acute otitis media and meningitis. *Streptococcus pyogenes* causes impetigo, acute tonsillitis, phlegmone and scarlet fever most frequently; whereas *Streptococcus agalactiae* is often an inhabitant of the human vaginal flora and can infect infants during delivery presenting fulminate sepsis, meningitis, or respiratory distress syndrome. In spite of the severity of the infections caused by these two latter species, they can be controlled, managed and overcome because of their sensitivity to some penicillins, cephalosporins and macrolides.

On the contrary, significant increase of resistance to antibiotics among *S. aureus*, CNS, *Enterococcus faecalis*, *Enterococcus faecium* and *S. pneumoniae* observed recently has created great therapeutic problems [1–10].

In this short review we focus the attention particularly on the β -lactam resistance and glycopeptide resistance of these “problematic” bacteria.

Mechanisms of action of β -lactam antibiotics

The site of action of β -lactams is the cell wall synthesis of bacteria [11]. Penicillins and other members of the β -lactam family kill bacteria by inactivating a set of transpeptidases that catalyze the final cross-linking reactions of peptidoglycan synthesis. Any penicillin derivate inhibits these enzymes by acting as a structural analogue, forming an irreversible penicilloyl-enzyme complex that is analogous to the transient acyl-enzyme formed during the normal transpeptidation reaction. Transpeptidases are difficult to assay and are usually detected and studied as penicillin-binding proteins (PBPs). Bacteria possess multiple transpeptidases that have different functions in the synthesis of the peptidoglycan during the cell cycle. Besides these physiologically important PBPs (PBPs with a high relative molecular mass – high M_r PBPs) bacteria contain one or more low- M_r PBPs that function as D-Alanine carboxypeptidases. Inactivation of low- M_r PBPs does not lead to killing of bacteria.

The consecutive steps of β -lactam action consist of binding of drug to cell receptor PBPs, inhibiting enzyme activities of PBPs [12], inhibiting phospholipid synthesizing enzymes, changing conformations of PBPs in membranes, changing membrane protein and lipid composition and integrity [13, 14], activating cell wall autolysins and activating murosomes [15]. As results of these activities inhibition of

transpeptidation of peptidoglycan molecules in cell wall synthesis, and deposition of uncrosslinked cell wall materials take place. The cells have structureless cross walls, thin sidewalls and enlarged cytoplasm volumes. Disturbance of selective permeability and active transport of bacterial membranes occurs, and proteins, lipids and DNA are released from the β -lactam treated bacterial cells [16]. Hyperactivity of autolysins and murosomes eventually lead to bacterial cell lysis and death.

Basic structural and physiological requirements for a β -lactam action are an intact β -lactam ring and the presence of side chains, growing bacteria, the presence of cell wall synthesis, accessible PBPs and high affinity of the drug to PBPs. In a hypertonic environment (e.g. urine in kidney, pyelon and bladder), damaged cell wall formation in bacteria leads to formation of spherical bacterial protoplasts from Gram-positive bacteria. These protoplasts are limited by the fragile cytoplasmic membranes. The pathogenetic role of these forms has still been questioned; although there are experimental evidences that abscesses in kidneys of mice infected with *S. aureus* and subsequently treated with some penicillin-contained staphylococcal protoplasts (Rozgonyi et al. unpublished data). If such protoplasts are placed in a normotonic environment they take up fluid, swell and may explode. Upon withdrawal of β -lactam from the environment they revert to cell wall synthesizing bacteria in a hypertonic environment or medium.

There are some basic factors affecting β -lactam effectiveness such as the degree of pH, binding time of the drug, the degree of affinity to PBPs and the competition ability for binding sites if more than one drug are applied (e.g. a penicillin and a cephalosporin).

Determination of β -lactam activities

As it later will be detailed, the simple disc diffusion test has a number of limitations to determine the true antibacterial activity of a given β -lactam derivate. Therefore, attempts have been made to use routinely a quantitative test such as macrodilution, microdilution or E-tests of β -lactams to measure exactly their minimal inhibitory concentrations (MICs) for a given strain of a species. All these together with the determination of the minimal bactericidal concentration are now equipped and convenient automated or semi-automated equipments, like Vitek (bioMérieux) are available to use panels or kits for the quantitative determination of the effectiveness of antimicrobials. However, all of these give static minute information about the effectiveness of the drugs. Considering the fact that all β -lactams are closely related structures, fine methods such as binding assay, growth kinetics, killing kinetics in

Bioscreen (Labsystem) and ultrastructural examinations have to be applied in order to make a more exact determination of the efficacy of these drugs against a given bacterial species.

Mechanisms of action of glycopeptide antibiotics

The glycopeptide antibiotics vancomycin and teicoplanin exert at least three consecutive or simultaneous effects on Gram-positive growing bacteria. They bind to the cell walls and cell membranes and alter the selective permeability of cells. Getting into the periplasmic space they inhibit the peptidoglycan synthesis by establishing complexes with D-Ala-D-Ala ends at the level of UDP-N-acetyl muramic acid pentapeptide formation. Both transglycosylation of disaccharides to form the polysaccharide backbone of cell walls and transpeptidation of pentapeptides to form peptide cross-bridges are inhibited either by a direct binding to transglycosylase and transpeptidase enzymes or by the above mentioned complexes preventing their access to their substrates. With similar mechanisms they eventually inhibit both RNA and DNA synthesis probably at the mesosomal-ribosomal complexes.

The effectiveness of glycopeptides also depends on a number of factors similar to those of β -lactams; however, the most critical is the penetrability through the cell wall cell-membrane barrier because of their highly hydrophobic and sterically large molecules. An outmost hydrophilic glycocalyx layer can hinder the glycopeptide penetration.

Resistance definitions

Resistance is an ability of bacteria to resist or tolerate the effect of an antimicrobial agent at therapeutic concentration. In the clinical aspect it is a quantitative measure, the MIC is higher than the maximal dose tolerable by the host.

Multiple resistance or *polyresistance* means that the bacteria are resistant simultaneously to a number of antibiotic agents with different chemical structures.

Cross resistance means that the bacterium is resistant to all derivatives coming from the same basic structure (e.g. macrolide antibiotics).

Extrinsic resistance means that the basis of the resistance is an extracellular factor excreted by the bacterium (e.g. β lactamase).

Intrinsic resistance means that the basis of the resistance is an intracellular known or unknown factor (e.g. binding protein).

β -lactam resistance in staphylococci

Antistaphylococcal β -lactams

Among the many β -lactams only few are considered as true antistaphylococcal ones; these are penicillin-G, -V, the aminopenicillins + β -lactamase inhibitors, methicillin, isoxazolympenicillins, 1st and 2nd generation cephalosporins. Bacteria of the genus *Staphylococcus* exhibit a variety of mechanisms to resist against β -lactams.

Extrinsic resistance

Modifying or destroying the molecules of β -lactams play outstanding roles in this resistance mechanisms.

Side chain splitting

Penicillin- and cephalosporin-acylases and -amidases produced by staphylococci can split the side chains of certain preparations of penicillins and those of cephalosporins (Fig. 1) resulting in a penicillin or cephalosporin nucleus lacking side chains [11], consequently possessing only a weak antistaphylococcal effect. This type of resistance is clinically not significant probably because of the much higher affinities of the antistaphylococcal penicillins and cephalosporins to their binding sites in bacteria than those of the enzymes for their substrates.

Hydrolysis of the β -lactam ring (penicillin resistance)

In contrast to the previous mechanisms, hydrolysis of the β -lactam ring of the penicillins and cephalosporins by staphylococcal β -lactamases is a clinically very significant resistance mechanism leading to a complete destruction of the β -lactam ring and to a loss of antibacterial activity of such drugs (Fig. 1). Staphylococcal β -lactamases have a narrow spectrum which means that they hydrolyze only penicillins very efficiently, except for β -lactamase-stable penicillins such as methicillin and isoxazoly- β -lactams. They fail to hydrolyze or very weakly hydrolyze cephalosporins and carbapenems. Staphylococci produce rarely β -lactamases constitutively, but the inductive production is very common. Both penicillins and cephalosporins may be good or bad β -lactamase inducers in staphylococci regardless of their antistaphylococcal activity.

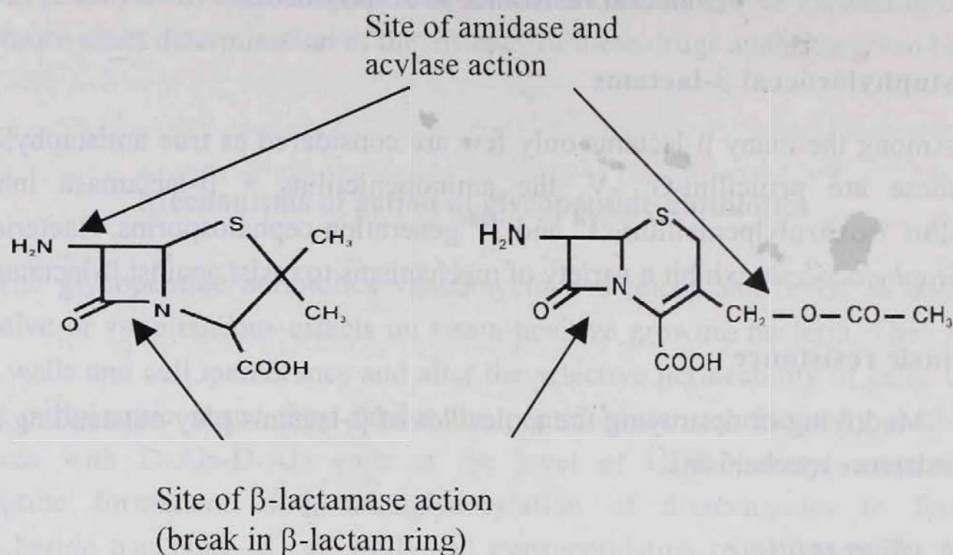


Fig. 1. Basic structures of 6-aminopenicillanic acid and 7-aminocephalosporanic acid and the sites of action of inactivating enzymes

There have been revealed approximately 50 types of staphylococcal β -lactamases on the basis of their amino acid sequences. They have been classified as types A, B, C and D on the basis of their interaction with antibodies raised against purified PC1 enzyme [17–21]. They show a variety of substrate profiles, inducibility, V_{max} ($\mu M/min$) and K_M (μM) [22]. Hungarian strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* showed 86%–88% resistance to penicillin (Table 1). The activity of staphylococcal β -lactamases can be inhibited sufficiently by clavulinic acid, sulbactam and tazobactam binding irreversibly to them with high affinity. These inhibitors protect hydrolysable penicillins from destruction when they are administered in combination [11, 19].

Genetic basis of staphylococcal β -lactamases

The structural gene is the *blaZ*; it codes for the β -lactamases in staphylococci. The *blaZ* is often found on large plasmids, but there are evidences for transposons containing *blaZ*. The transposons, however, may not have specific sites for insertion and complete plasmids are capable of integrating into the chromosome. It is to be expected that the gene will be

found in many different chromosomal locations. Two of the regulatory genes *blaI* and *blaR1* are found together with *blaZ* on large plasmids. The *blaI* is the gene for the classical repressor protein *BlaI*. This protein binds specifically to the *blaZ* operator region. The *blaR1* is the gene for a putative signal sensor-transducer protein. Another regulatory gene, *blaR2* is always a chromosomal gene. For most isolates of β -lactamase producing staphylococci the enzymes are produced inducibly, only the D-type enzyme in one strain is produced constitutively. The model for induction is that the inducer's β -lactam binds to the C terminus region of *BlaR1*, causing a signal to be passed to the *BlaI* protein. As a result of the signal the *BlaI* binds no longer to the operator region, and so the transcription of *blaZ*, *blaR1* and *blaI* may start. Both the synthesis of the β -lactamases and the regulatory proteins are increased [19, 23, 24].

Detection of β -lactamases

Many tests for β -lactamase production, including either chromogenic reactions or detection of the destruction of antibiotic activity have been proposed [23]. Penicillin is the antibiotic recommended for both disc diffusion and dilution antimicrobial susceptibility testing. The disc diffusion sensitivity test can be done by using a 10- μ g penicillin disc in an agar-diffusion sensitivity test. β -lactamase producing strains will show no or very narrow inhibition zones around the disc depending on the length of incubation. Strains that have a MIC greater than 0,03 μ g penicillin/ml must be confirmed as susceptible by a β -lactamase detection test. The chromogenic methods are faster and more convenient. They are divided into those in which the hydrolysis of the β -lactam itself engenders a color change and those in which this change depends on a linked reaction. The first type test uses the nitrocephin as a chromogenic cephalosporin substrate, which changes on hydrolysis from yellow to pink. Nitrocephin can be used in a test tube assay or as a disc test. Linked detection systems include the iodometric and acidimetric methods. The product of the hydrolysis of β -lactams reduces iodine to iodide, this way a decolorisation of starch-iodine occurs.

Acidimetric test is based on the fact that opening the β -lactam ring generates a free carboxyl radix, and this acidity can turn bromocresol purple from violet to yellow in an unbuffered system [17, 19, 23, 25].

Table I

*Susceptibility of isolates of most frequent Staphylococcus species cultured from patients examined/treated at the clinics of Semmelweis University of Medicine, Budapest, Hungary from January 1997 to December 2000**

Antibiotic	No. of isolates tested	Percentage		
		Sensitive	Intermediate	Resistant
<i>Staphylococcus aureus</i>				
Penicillin	3036	13.9	0	86.1
Oxacillin	2996	73.1	1.3	25.6
Vancomycin	2940	99.6	0.1	0.3
Teicoplanin	2203	91.4	5.8	2.8
<i>Staphylococcus epidermidis</i>				
Penicillin	1857	9	0	91
Oxacillin	1816	44.8	1.1	54.1
Vancomycin	1806	99.5	0.2	0.3
Teicoplanin	1460	92.9	5.1	1.9
<i>Staphylococcus haemolyticus</i>				
Penicillin	271	16.6	0	83.4
Oxacillin	266	66.2	0.4	33.4
Vancomycin	263	99.2	0	0.8
Teicoplanin	207	83.1	8.2	8.7
<i>Staphylococcus hominis</i>				
Penicillin	269	15.2	0	84.8
Oxacillin	267	50.9	0.7	48.3
Vancomycin	265	100	0	0
Teicoplanin	220	94.1	4.1	1.8

* Results of disc diffusion test

Intrinsic resistance

Staphylococci have different ways to become intrinsically resistant to β -lactam antibiotics. The primary targets of β -lactam antibiotics are the PBPs. These enzymes (PBPs) are responsible for the polymerization of peptide moieties of the peptidoglycan chains, which in staphylococci are cross-linked by a

characteristic pentaglycyl side chain [26]. Staphylococci can alter their PBPs either by mutation, or more efficiently and clinically more relevant, by acquisition of a foreign DNA element, a transposon, coding for methicillin resistance (MR). MR determinant element is called *mec*. *Mec* determinant confers to the staphylococci an intrinsic cross-resistance against all β -lactams regardless of their accessibility by β -lactamases.

Phenotypic characteristics of MR

Two main phenotypes of resistance occur among MR staphylococci: homogenous resistance (class 4) and heterogeneous (classes 1–3). Clinical isolates have been divided into four classes according to MR expression by Tomasz and co-workers [27] as follows: class 1 (MIC 1.5–3.0 $\mu\text{g}/\text{ml}$ for $\geq 90\%$ of population, HRS highly resistant subpopulation, 10^{-8} – 10^{-6}), class 2 (MIC 6.0–12.0 $\mu\text{g}/\text{ml}$ for $\geq 90\%$ of population, HRS, 10^{-5} – 10^{-4}), class 3 (MIC ≥ 50 $\mu\text{g}/\text{ml}$ for majority of population, HRS, 10^{-3} – 10^{-1}), class 4 (homogeneously highly resistant, MIC > 400 $\mu\text{g}/\text{ml}$). Until now the molecular nature of the heteroresistance was not satisfactorily resolved.

Mutation to MR is accompanied by multiresistance of cocci and MR is phenotypically suppressed at elevated temperature (42°C) but is genotypically stable [28]. It is expressed best at 30°C on 2% NaCl Mueller-Hinton agar, when incubation lasts for 48h.

Properties of methicillin-resistant staphylococci

They have higher membrane lipid content in their membranes and lysozyme resistant peptidoglycans [29, 30]. They have longer generation time, consequently they show lower growth rate than their methicillin-sensitive (MS) counterparts. MR cells show retarded cell separation [31]. In experimental infections they need higher number of viable counts to cause LD50 but they show higher index of organ persistence than their MS counterparts. MR cocci show high virulence in immunocompromized hosts [32].

Genetic basis of methicillin-resistance

The *mec* determinant resides on a DNA element of more than 30 kb that is absent in susceptible staphylococci. This DNA element seems to integrate in the genome of *S. aureus* at a specific site, between the *spa* (protein A) and *purA* (adenine requirement) genes. The *mecA* gene is only a small part of this

element. It produces a low-affinity 76 kDa PBP2'. The core sequences of the *mec* element consist of this *mecA* gene and a hypervariable segment of 3'DNA that ends in an insertion-sequence like element. Upstream of *mecA*, its operator and promoter regions separate a regulatory element, which contains the *mecR1* and *mecI* genes. The gene *mecI* codes for a repressor protein (*MecI*). This protein has strong similarity to *BlaI*, the repressor of the *blaZ* gene. The product of the *mecR1* gene, the *MecR1* is similar to *BlaR1*, which is involved in the induction of β -lactamase enzymes. Because of their great similarity to *MecR1* and *MecI*, the regulatory elements *BlaR1* and *BlaI* can also regulate *mecA* transcription. In the absence of *mecI*–*mecR1* and β -lactamase regulatory elements, there is a constitutive PBP2' production, but some strains are strongly repressed and produce PBP2' only after induction. Methicillin and oxacillin seem to be not enough strong inducers. The resistance is established very slowly, full induction on methicillin-containing plates is seen only after 48 hours. Many clinical strains that carry the *mecI* and *mecR1* genes produce PBP2' constitutionally in high amounts, they have point mutations or deletions that inactivate the repressor.

The expression of the *mecA* gene largely depends on the activity of genes termed factors essential for methicillin-resistance (*fem*). The *femA* and *femB* is involved in the formation of the peptidoglycan pentaglycine interpeptide bridge. Mutants lacking *femA* or *femB* are able to attach only two or three glycines to the cross bridge. The precise role of these two cytoplasmic proteins is unknown, but it is known that every insert in *femA* and *femB* abolish methicillin-resistance completely [26, 33–36].

The first MRSA containing the *mec* determinant was isolated in 1960. At that time the incidence of MRSA was less than 0.1 percent of all isolates, but now they are spread all over the world, mainly in hospitals where is a constant strong antibiotic pressure. Clonal analysis of MRSA and other *Staphylococcus* species supports the hypothesis that there may be a dissemination of the *mec* determinant by horizontal transfer [26]. The MR frequency in Hungarian *Staphylococcus* strains is shown in Table 1 and in Fig. 2. The results of molecular detection of methicillin resistance are shown in Fig. 3. The MIC range of oxacillin for *Staphylococcus aureus* strains proved to be wide, in contrast to that for *Staphylococcus epidermidis* strains, which required high MICs to inhibit growth.

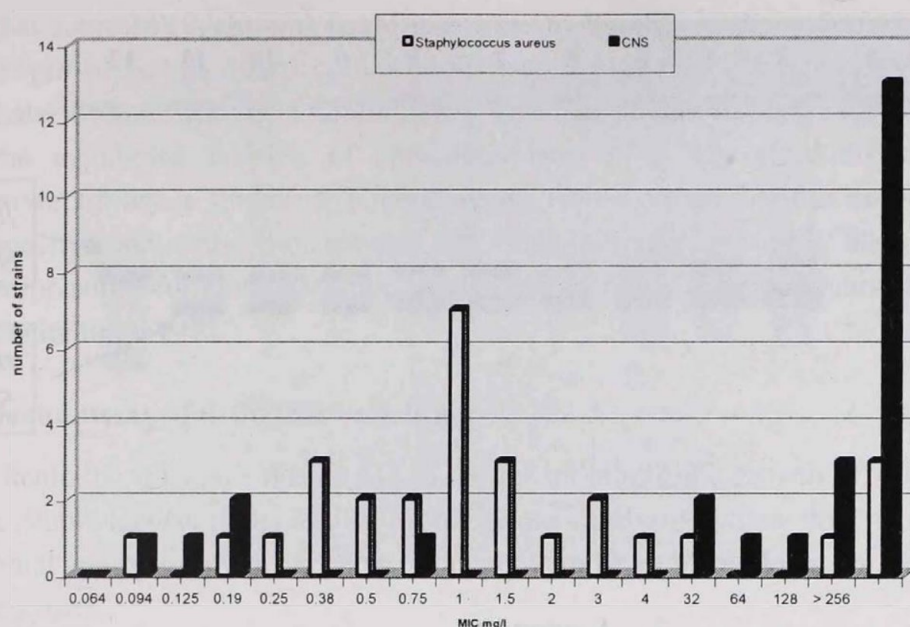
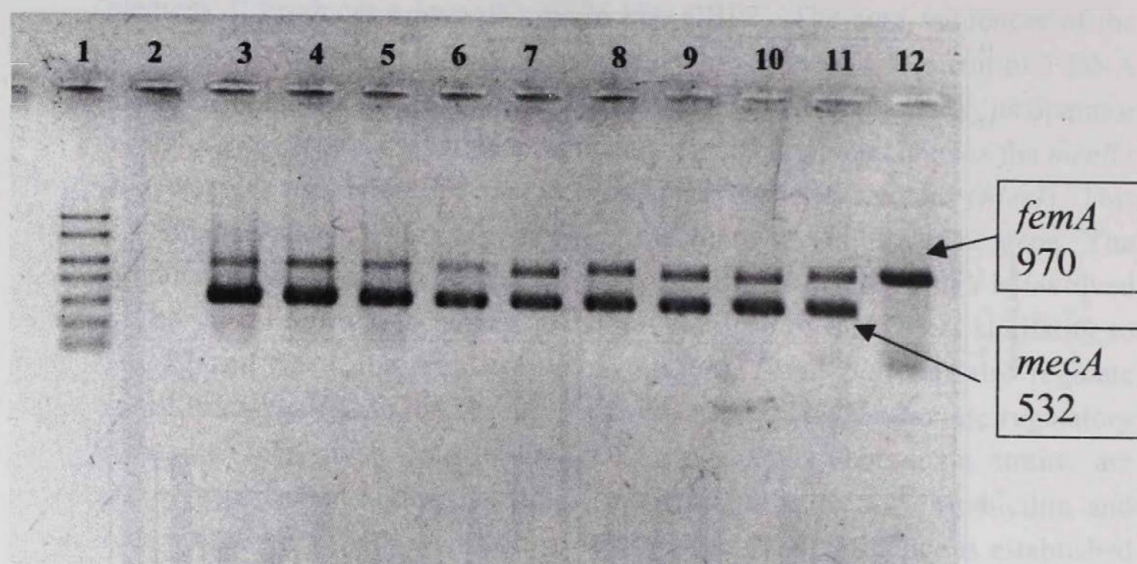


Fig. 2. Distribution of the minimal inhibitory concentrations of oxacillin for *Staphylococcus aureus* and CNS strains isolated in the year 2000 from patients treated at the clinics of the Semmelweis University, Budapest, Hungary

Detection of MR

The broth macrodilution method has proven to be the most appropriate for quantitative detection of MR. However, it is extremely laborious and time consuming. Therefore, broth microdilution is also accepted in duplicate rows. To screen MR colonies, seeding 6 μ g/ml oxacillin agar is recommended. For the routine susceptibility testing, the NCCLS has approved 1 μ g oxacillin disc, but in many countries higher oxacillin contents have been applied. Using any of these methods the following requirements are common and accepted: high ($\leq 10^6$ CFU/ml) inoculum, 2% NaCl in the medium, low (30–35°C) incubating temperature and prolonged incubation time (48h) [37–40]. Of course, if the MR colonies appear earlier one can report MR.



Legend

- | | |
|--------------------------|--|
| 1. marker | 7. <i>S. aureus</i> 2794 |
| 2. no sample | 8. <i>S. aureus</i> 2260' |
| 3. <i>S. aureus</i> 2260 | 9. <i>S. aureus</i> 3282' |
| 4. <i>S. aureus</i> 3282 | 10. <i>S. aureus</i> 2724' |
| 5. <i>S. aureus</i> 2724 | 11. <i>S. aureus</i> BB270 positive control |
| 6. <i>S. aureus</i> 1978 | 12. <i>S. aureus</i> ATCC 29213 negative control |

Fig. 3. Detection of the *mecA* and *femA* genes by multiplex PCR from *Staphylococcus aureus* isolates

Borderline oxacillin resistance

Borderline strains may be divided into two categories on the basis of whether they have *mecA* gene. Borderline strains that contain *mecA* gene are extremely heterogeneous methicillin resistant strains that produce PBP 2a [41]. Borderline strains that do not contain *mecA* gene can be differentiated phenotypically from extremely heterogeneous *mecA* positive strains, as highly resistant clones do not occur. This borderline resistance has been hypothesized to result from modification of normal PBP genes or overproduction of staphylococcal β -lactamase. Tomasz et al. reported alterations of penicillin binding by PBPs 1, 2 or 4 in β -lactamase-negative, *mecA*-negative borderline clinical strains [42]. These binding alterations are the result of point mutation in the penicillin-binding domain [43]. Overexpression of PBPs also can produce low-level resistance [44]. Borderline strains that are β -lactamase hyperproducers, and show relatively high levels of β -lactamase activity in biochemical assays, and exhibit a lowering of MICs into the susceptible range upon addition of β -lactamase inhibitors, or upon elimination of the β -lactamase plasmid [45]. As early as in 1968 Rozgonyi and Rédei reported first

that some MRSA strains could inactivate methicillin in their stationary phase of growth, while oxacillin both in the exponential and stationary phases [46]. Later Montanari et al. found a new β -lactamase that hydrolyses methicillin in the membrane fraction of clinical isolates of *S. aureus* with borderline susceptibility to this drug. Kinetic assays demonstrated that this second more specific inducible β -lactamase, the methicillinase is more likely to be responsible for the borderline phenotype than an increased amount of the penicillinase [47].

Other mechanisms of β -lactam resistance

Penicillin tolerance means that the drug can inhibit the growth of, but cannot kill the staphylococci. This results from failure of the β -lactam drug to activate murosomal and/or autolytic enzymes in the peptidoglycan break down, or the lack of these enzymes.

An overproduction of structured cell walls and/or alteration in the membrane lipid amount and composition may also lead to a high tolerance to β -lactams [30].

Another resistance mechanism is due to the absence of some penicillin receptor in the membrane. It occurs as a result of chromosomal mutation.

Glycopeptide resistance of *Staphylococcus aureus*

Since the emergence of MRSA, the use of the glycopeptide vancomycin and teicoplanin has been the only uniformly effective treatment for such staphylococcal infections. The recent appearance of glycopeptide-resistant coagulase-negative staphylococci [48–53] has heightened concern about whether or not *S. aureus* could acquire glycopeptide resistance. The emergence of such resistance could produce morbidity and mortality similar to that caused by *S. aureus* infections in the pre-antibiotic era.

The first well-documented clinical infection caused by an intermediate glycopeptide resistant strain of *S. aureus* was reported from Japan in 1996 with a MIC of 8 $\mu\text{g/ml}$ [54]. According to the NCCLS, this strain is certainly not susceptible (defined as having a MIC of $<4 \mu\text{g/ml}$), but is also not yet resistant (defined as having MIC of 32 $\mu\text{g/ml}$ or greater). This type of *S. aureus* was therefore described as vancomycin intermediate sensitive *S. aureus* (VISA), or more particularly to glycopeptides (GISA). Since this first isolation, there have been further three cases of clinical infections due to strains with reduced vancomycin susceptibility reported from the United States in Michigan, New Jersey, and New York [55].

In Europe, no *S. aureus* strains with reduced susceptibility have been reported yet. Lessing et al. [56] conducted a retrospective audit of altogether 137 episodes of blood stream infection due to *S. aureus* from London, United Kingdom. The authors re-tested 32 stored *S. aureus* stock-cultures (25 MSSA and 7 MRSA), but found no isolates with reduced susceptibility, or resistance to vancomycin. Despite relatively early reports about clinical isolates of glycopeptide resistant coagulase-negative staphylococci [48–52], moreover the successful *in vitro* transfer of vancomycin resistance genes from *E. faecalis* into *S. aureus* [57], as well as the emergence of GISA strains, glycopeptide fully resistant (MIC ≥ 32 $\mu\text{g/ml}$) *S. aureus* primary clinical isolate has not been reported yet. Although there is a great concern about the emergence of such strains since from one of the U.S. isolates of GISA a more resistant sub-population (MIC = 16 $\mu\text{g/ml}$) could be easily selected *in vitro* by population analysis in culture media with a frequency of 10^{-3} . After the selection, this sub-population still remained heterogeneous and contained approximately 10^5 bacteria capable of growing even at a vancomycin concentration of 16 $\mu\text{g/ml}$ [58]. It can be easily predicted that such a selection may take place *in vivo* during vancomycin and teicoplanin therapy of infections due to GISA strains.

With Professor Waldvogel's words [59]: "*Et tu mi, fili?*" (means "You, my son, as well?"). This was Julius Caesar's outcry when, surrounded by conspirators ready to stab him to death, he discovered among them Brutus, his adopted son. Caesar's outcry was one of surprise, but also one of recognition that Brutus's betrayal could have been predicted.

Possible mechanisms of glycopeptide resistance of S. aureus

The exact mechanism(s) of glycopeptide resistance has not been revealed yet. Significant amount of data have accumulated, however, allowing to predict the resistance mechanism(s). Glycopeptide resistance may have emerged in *S. aureus* because of interspecies transfer of resistance genes (i) or selection of resistant mutants as a result of prolonged antimicrobial therapy (ii).

Ad (i), the ability of Gram-positive micro-organisms to acquire glycopeptide resistance genes became a matter of concern with the emergence of vancomycin-resistant enterococci (VRE) [60], and vancomycin resistance genes have been transferred from VRE strains to *S. aureus* *in vitro* [61]. However, none of the clinical GISA isolates have had *vanA*, *vanB*, *vanC1*, *vanC2*, or *vanC3* genes yet [62], suggesting that interspecies transfer of resistance genes from VRE is not the *in vivo* mechanism by which glycopeptide resistance developed in these *S. aureus* isolates.

Ad (ii), certain common factors in the cases of the two independent U.S. cases suggest that cellular modification due to prolonged vancomycin exposure was probably responsible for the emergence of glycopeptide resistance in these isolates [63]. In both cases, patients received multiple prolonged courses of vancomycin for severe MRSA infections. The patients' MRSA isolates, as well as their GISA had similar MIC to antimicrobials other than vancomycin. In addition, *S. aureus* isolates that had intermediate vancomycin resistance had increased extracellular surface material associated with the cell walls of the cocci. This finding is compatible with that observed in *S. aureus* with intermediate glycopeptide resistance induced in vitro [64–66]. The thickened extracellular surface material has been shown to sequester vancomycin [64] and to reduce the susceptibility of *S. aureus* to vancomycin [66]. The exact mechanism, however, has not been determined, yet, but these data suggest that it emerged through the selection of naturally occurring resistant mutants during prolonged exposure to vancomycin. The phenomenon that teicoplanin resistant strains of different *Staphylococcus* species are sensitive to vancomycin has also not been sufficiently explained [53]. Other observations are also pursuing the hypothesis that PBPs are overproduced, allowing them to compete with vancomycin for binding to peptidoglycan precursors [66, 67].

Elucidation of these mechanisms will be essential for the development of effective therapeutic agents in the future.

Detection of glycopeptide resistant S. aureus

The emergence of GISA strains worldwide suggests that *S. aureus* strains with full resistance to vancomycin may eventually emerge. These episodes emphasize the need to enhance laboratory capacity at the hospitals and national levels to recognize these strains. Furthermore, an effective international co-operation with exchange of national data would seem also beneficial for all parties.

Identification of the species should be carried out based on the standard reference methods [68]. Data suggest that antibiotic susceptibility tests performed by the disc-diffusion method may differ greatly from those carried out by broth microdilution techniques [63]. On disc-diffusion testing, the two U.S. isolates were read as susceptible to vancomycin at 18 and 17 mm of zone of inhibition, respectively. In contrast, they were intermediately susceptible to vancomycin with a MIC of 8 $\mu\text{g/ml}$ when tested with broth microdilution [63]. Although, data concerning glycopeptide agar-gradient-diffusion susceptibility tests (E-test) in case of GISA strains as compared to broth micro-dilution tests are not readily available, it seems that vancomycin susceptibility test should be performed with one of the above quantitative methods at least in case of high risk for vancomycin resistance [56]. All the other

antimicrobials should be tested according to the guidelines of NCCLS with the quantitative methods whenever it is possible. It is especially true for oxacillin (methicillin). If available, *mecA* PCR test should also be performed.

Although not routinely done, population analysis profile of *S. aureus* strains with intermediate susceptibility should be also performed as described by Edmond et al. [69], and Siedradzki et al. [58] to detect heterogeneous resistance. Although accumulated data about clinical isolates of GISA strains do not support the idea of the transfer of vancomycin resistance genes from VRE, because of the low number of cases this possible mechanism of resistance cannot be excluded. Consequently, it seems advisable to follow up all the GISA isolates for *vanA*, *vanB*, *vanC1*, *vanC2*, or *vanC3* genes by means of PCR techniques in suitable reference laboratories [60–62]. Molecular epidemiology studies on the isolated GISA strains (PFGE, DNA fingerprinting) should be performed in reference laboratories to assist epidemiological surveillance [58, 59, 63].

Possible origin of glycopeptide resistance

The selection of resistant mutants under the pressure of prolonged glycopeptide exposure on the normal staphylococcal and enterococcal flora seems to be the most probable origin. It may take place both *in vitro* – that has been proven – and *in vivo* – that remains to prove.

The extensive use of 3rd generation oral cephalosporins worldwide including Hungary has contributed indirectly to this selection process since staphylococci and enterococci are inherently resistant to these drugs.

Acquired resistance may be established by intra- and interspecies transfer of vancomycin resistance genes from enterococci. This has occurred so far *in vitro* only.

In Hungary phenotypically vancomycin resistant *S. aureus* strains have rarely been isolated (Table 1 and Fig. 4). However, teicoplanin resistant strains have been found in both *S. aureus* isolates and many other *Staphylococcus* species [53].

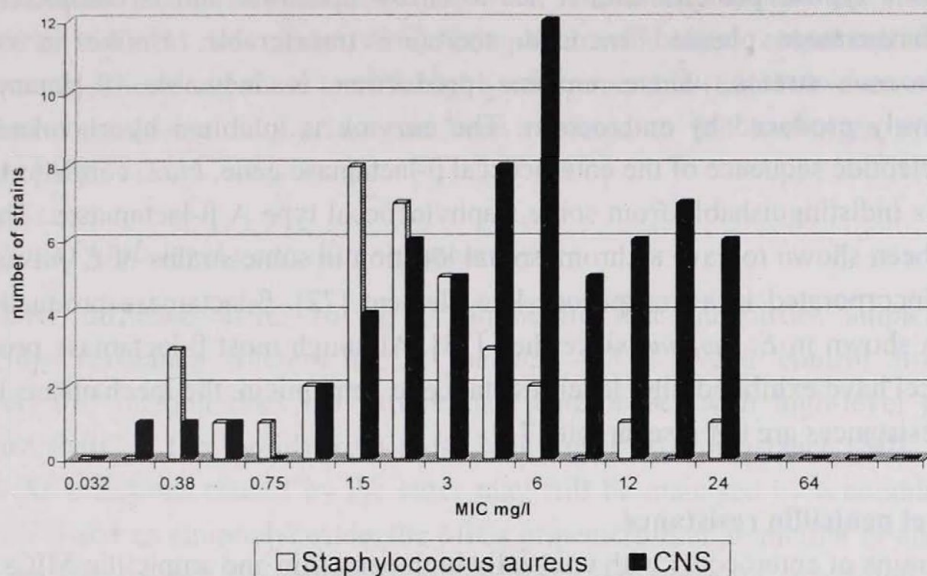


Fig. 4. Distribution of the minimal inhibitory concentrations of teicoplanin for *Staphylococcus aureus* and CNS strains isolated in the year 2000 from patients treated at the clinics of the Semmelweis University, Budapest, Hungary

β -lactam-resistance in enterococci

Mechanisms of β -lactam resistance

Intrinsic resistance

Enterococci possess relative intrinsic (naturally inherited) resistance against penicillin-G, -V, as well as the 2nd and 3rd generation cephalosporins. This resistance is attributable to one or more of the below described mechanisms. The drug cannot access the target, mostly because the cell membranes of enterococci contain high amount of lipids, furthermore the surfaces of the cocci appear to be highly hydrophobic. There may be an increased efflux of the drug from the periplasmic space, which may decrease the concentration of β -lactams to an insufficient level. Then, autolytic enzymes of the enterococcal cell walls may be deficient; consequently they fail to cause the death of the cocci. Enterococci grow relatively slow, and this phenomenon provides a relative protection against β -lactam antibiotics.

β -lactamase production

β -lactamase production in *E. faecalis* was first detected in 1983 [70]. The enzyme is a typical penicillinase. It has a narrow spectrum and is completely cell-bound, furthermore plasmid encoded, therefore transferable. Unlike in case of *Staphylococcus* strains, where enzyme production is inducible, β -lactamase is constitutively produced by enterococci. The enzyme is inhibited by clavulanic acid [71]. Nucleotide sequence of the enterococcal β -lactamase gene, *blaZ*, confirms that the enzyme is indistinguishable from some staphylococcal type A β -lactamases. The gene has also been shown to have a chromosomal location in some strains of *E. faecalis* and may be incorporated in a transposone-like element [72]. β -lactamase production has also been shown in *E. faecium* since then [73]. Although most β -lactamase producing enterococci have exhibited high level resistance to gentamicin, the mechanisms leading to both resistances are not inseparable [74].

High-level penicillin resistance

Strains of enterococci with unusually high penicillin and ampicillin MICs, in the absence of β -lactamase production, are increasingly being reported [75, 76]. This high level resistance is thought to result from overproduction of the slow reacting PBP 5, a normal PBP of enterococci, which can substitute for other PBPs in the cell wall synthesis [77]. High-level penicillin resistance may result in a loss of synergy with gentamicin, although in case of some strains this may be overcome with high concentration of penicillin [78].

Detection of β -lactam resistance

There are some important differences among species of the *Enterococcus* genus regarding penicillin, aminoglycoside and glycopeptide resistance patterns. Therefore, enterococci causing serious infections should be fully identified. The minimum requirement is the proper identification of the species isolated most frequently from clinical specimens: *E. faecalis*, as well as *E. faecium*. Classical methods (growth characteristics, colonial morphology, conventional biochemical reactions) aid the determination of different species of the genus. A number of commercially available tests also provide sufficient identification of *Enterococcus* strains [79].

Detection of β -lactamase production

When detecting β -lactamase production, one should pay attention to the fact that β -lactamase producing enterococci exhibit a marked inoculum effect, that is, penicillin resistance may not be demonstrated unless a high inoculum (e.g. 10^7 CFU/ml) is used. There are commercially available rapid tests for the detection of the enzyme. In these

tests, chromogenic substrates of the β -lactamase enzyme are incorporated into discs, which should be placed onto the plate culture of enterococci. Result of the test is then read based on the change of color of the substrate. Studies also indicate that the classical acidimetric test was less sensitive as compared with these colourimetric assays. Consequently, the use of these tests is highly recommended [80]. However, a strain of β -lactamase producing *E. faecalis* has been reported to fail hydrolyze nitrocephin substrate [81].

Detection of high-level penicillin resistance

Disc diffusion using 10 μ g of ampicillin disc identifies ampicillin and amoxicillin resistance when using *E. faecalis* ATCC 29212 control strain [82]. However, this method does not differentiate enterococci with high-level penicillin resistance (MIC $\geq$ 128 μ g/ml) from those with lower level resistance (MIC = 16–32 μ g/ml). As infections caused by the latter may still be managed by a combination of penicillin-G and an aminoglycoside, the MICs of penicillin or ampicillin or amoxicillin should be determined for enterococci causing life-threatening infections, such as endocarditis, sepsis etc. Broth microdilution method is the gold standard for the measurement of MIC of penicillin, ampicillin or amoxicillin in case of enterococci. However, the E-test also seems to be a reliable method, and an acceptable option for the detection of MICs. In a study, its quantitative accuracy compared with agar dilution results was 95% for *Enterococcus* spp. The test procedure is easy to perform, reproducible, and quantitatively and qualitatively accurate [83, 84]. Another option for the detection of high-level penicillin resistance is using commercially available panels that also allow sufficient identification of the isolate based on biochemical patterns, as well as reliable susceptibility profile for selected antimicrobial drugs (e.g. ampicillin, vancomycin) utilizing the positive breakpoint method [85]. The major advantage of these assays is that they parallel provide species identification and susceptibility data at the same time, although misinterpretation of species, and errors determining susceptibility may be observed in such tests. The frequency of β -lactam-resistant enterococcal strains in Hungary is shown in Table 2.

If the patient has an allergy to penicillins, or if the enterococcal strain is resistant to ampicillin, which leads to a loss of synergism with aminoglycosides, glycopeptides, such as vancomycin or teicoplanin are the antibiotics of choice. During the past 15 years the majority of *E. faecium* strains have become resistant to ampicillin in many countries [76, 86], and a number of strains have acquired high-level resistance to aminoglycosides [77]. This has apparently occurred via the acquisition of *Tn4001*-like transposons carrying the determinant for the bi-functional AAC (6')-APH (2'') enzyme and originating from staphylococci [87].

Table II

*Susceptibility to some β -lactam antibiotics and vancomycin of Enterococcus spp. and Streptococcus pneumoniae isolates cultured from patients treated at the clinics of the Semmelweis University of Medicine, Budapest, Hungary in the years 1997–2000**

Antibiotic	No. of isolates tested	Percentage		
		Sensitive	Intermediate	Resistant
<i>Enterococcus faecalis</i>				
Penicillin-G	845	0	21	79
Amoxicillin / Ampicillin	881	89	2	9
Piperacillin	705	90	2	8
Imipenem	815	94	6	0
Vancomycin	857	98.7	0.4	0.9
<i>Enterococcus faecium</i>				
Penicillin-G	71		21.1	78.9
Amoxicillin / Ampicillin	73	61.6	9.6	28.8
Piperacillin	61	70.0	1.6	28.4
Vancomycin	71	90.1	–	9.9
<i>Streptococcus pneumoniae</i>				
Penicillin-G	907	61	13.7	25.4
Amoxicillin / Ampicillin	842	84.8	3	12.2
Cefotaxime	751	92.7	2.4	4.9
Vancomycin	399	99	1	–

* Results of disc diffusion method

Glycopeptide resistance in enterococci

Resistance to glycopeptides in enterococcal clinical isolates was reported first by Leclercq et al. [88] and Shlaes et al. [89] and has been increasingly reported in recent years. By molecular typing of VRE from these outbreaks, it is possible to detect both the spread of particular strains, which is presumably related to poor hospital hygiene (exogenous infections) [90–93], and probably endogenous infections, as indicated by the variety of strains [94]. In 1993 the Centers for Disease Control [95] reported that the total resistance to glycopeptides in enterococci from nosocomial infections in U.S. hospitals increased from 0.3% in 1989 to 7.9% in 1993, enterococcal

strains from patients in intensive care units showed an increase of resistance to these antibiotics from 0.4% to 13.6% in the same period of time.

Not only because of their different pathogenic potency but also because of their variety in antibiotic resistance patterns, it is important to identify enterococci up to the species level as mentioned above [96]. *E. faecium* is more resistant to several antibiotics than *E. faecalis*, and resistance to glycopeptides is also more frequent in *E. faecium*. Enterococcal species occurring in animals preferentially can also have acquired resistance to glycopeptides [97].

Identification criteria for enterococci are that they are catalase-negative, gram-positive cocci, bile-esculin and pyrrolidonylarylamidase positive, and that they grow in 6.5% NaCl broth and possess the Lancefield group D antigen [98].

Up to now six genotypes of glycopeptide resistance are known: *vanA*, *vanB*, *vanC*-1, *vanC*-2 [97, 99], *vanD* [100] and *vanE* [101]. The *vanA* genotype is inducible and the most widespread in enterococci. It is mainly found in *E. faecium*, but also occurs in *E. faecalis*, *E. avium*, *E. durans*, and other species. This type is also the most significant one with respect to the chemotherapy options of VRE infections, since it mediates high-level resistance and cross-resistance to both vancomycin and teicoplanin used in human medicine and also to the animal food additive glycopeptide avoparcin. The *vanB* genotype expresses variable and inducible resistance to vancomycin but not to teicoplanin. The *vanC* genes are characterized by low-level resistance to vancomycin and it is specific to *Enterococcus gallinarum*, *E. casseliflavus* and *E. flavescens* [102, 103]. The *vanD* genes encode variable resistance levels to vancomycin and teicoplanin [100]. The *vanE* genes are present in a single isolate of *E. faecalis* BM4405 and susceptible to teicoplanin and resistant to low level of vancomycin [101]. The *vanA* genotype is encoded by the *vanA* gene cluster, which in some strains is integrated into the transposon *Tn1546*. This transposon can be transferred to other enterococcal strains and species at high frequency when located on conjugative plasmids.

The VanA, VanB and VanD ligase synthesize the depsipeptide D-Ala-D-Lac, instead of D-Ala-D-Ala. In VanA- and VanB-type strains three other enzymes are required for resistance: the VanH dehydrogenase reduces pyruvate to D-Lac [104], whereas the VanX D,D-dipeptidase and the penicillin G-insensitive VanY D,D-carboxypeptidase [105] prevent synthesis of UDP-MurNAc-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (pentapeptide [Ala]). The VanA and VanB phenotypes of resistance, but not the VanD phenotype, are generally transferable to other enterococci by conjugation. Acquired glycopeptide resistance in enterococci is based on a modification of the target of these antibiotics, the D-alanyl-D-alanine end group of the disaccharide pentapeptide cell wall precursor. The C-terminal D-Alanine is replaced by a D-lactate. The corresponding genetic determinants encoding the ligase, dehydrogenase, D,D-

dipeptidase, and D,D-carboxypeptidase as well as two regulatory genes encoding the response regulator and the sensor/Histidine kinase are located on transposons [106, 107, 108]. The *vanA* protein, a cytoplasmic membrane protein of 39 kDa, is the main component of this resistance mechanism [104, 109, 110]. The VanA phenotype is inducible by vancomycin and teicoplanin and confers high MICs to the three glycopeptides. Strains of the VanB type have acquired inducible resistance to various levels of vancomycin but not to teicoplanin [111]. Constitutive low-level resistance to vancomycin (VanC type) is an intrinsic property of motile enterococci, *E. gallinarum*, *E. casseliflavus* and *E. flavescens* [102, 103, 112].

The actual prevalence of vanC type resistance might even be higher since phenotypic detection of low-level resistance is difficult [113, 114]. Several recent outbreaks of VRE [115, 116] emphasize the need for laboratories to be able to detect the various types of resistance.

Molecular detection of glycopeptide resistance

The PCR method has been extensively applied in bacteriological diagnosis of VRE infections [117]. It has been used for species identification of infectious agents [118–120] and specific detection of antibiotic resistance genes [57]. In the year 1999 our workgroup could detect by PCR the first glycopeptide resistant *E. faecalis* in Hungary [121]. The result is shown in Fig. 5.

Emergence of VRE outside the hospitals

VRE has been detected not only inside the hospitals but also outside them evidencing by their isolation from the wastewater of sewage treatment plants [122, 123]. Some of these plants were located in rural areas of Germany [122] where there has been no hospital in the corresponding town or where the use of glycopeptides in the local hospitals has been exceptional and intravenous.

Glycopeptides have been also used outside the hospitals, as so-called “growth promoters” in animal husbandry. For about 20 years the glycopeptide antibiotic avoparcin has been available as a feed additive in many western European countries and in Australia, although not in the U.S. or Canada. Avoparcin has a glycopeptide chemical formula similar to that of vancomycin and teicoplanin, which have been used in human medicine [124]. This has suggested that avoparcin could have been responsible for the selection of glycopeptide-resistant enterococci, since it is not absorbed from the gut, therefore it may be present at high concentration for a long period of time. In addition, the mechanism of resistance to avoparcin, similar or equivalent to that for vancomycin and teicoplanin [104, 109, 110], is likely to exist.

Avoparcin is used as a feed supplement for broiler chickens, turkeys, pigs, beef and dairy cattle, calves, goats and sheep, too.

Outlook

Experimental conjugative transfer of glycopeptide resistance has been done with *E. faecalis* [116] and also between *E. faecalis* and *S. aureus* [61]. Between *S. aureus* and enterococci horizontal gene transfer is possible under natural conditions, as demonstrated by the similarity of transposons encoding aminoglycoside resistance in *S. aureus* and *E. faecalis* [87] and by the characterization of *E. faecalis* strains carrying a β -lactamase whose origin was *S. aureus* [125, 126]. The recent detection of the *vanA* gene in *Corynebacterium haemolyticum*, *Lactococcus* and *Listeria species* [96, 127, 128], as well as that of *vanB* in *Streptococcus bovis*, indicates the potential for the spread of glycopeptide resistance to other organisms.

Phenotypically vancomycin-resistant strains of different *Enterococcus* species have been isolated in Hungary, too, and their incidence is relatively high (Table 2). However, *vanA* positive *E. faecalis* strains proven by PCR technique were isolated from two clinical cases so far [121, 129].

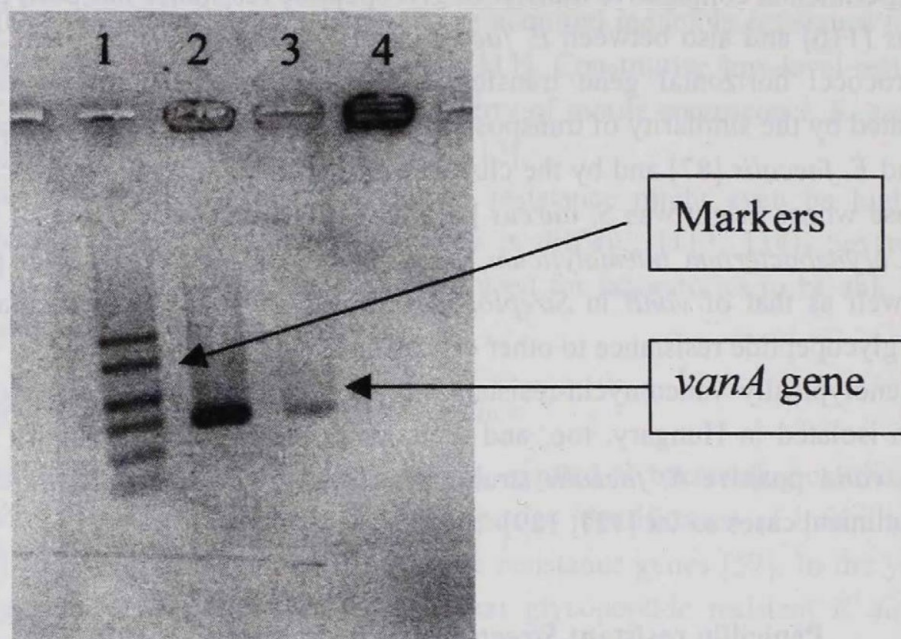
Penicillin resistant *Streptococcus pneumoniae* (PRSP)

Mechanism of β -lactam resistance

In the relatively few bacterial species in which β -lactamases are unknown to exist the development of high M_r PBPs with decreased affinity for β -lactam antibiotic may be one of the mechanisms that can provide increased resistance. The most spectacular example of PBP-mediated resistance is found in *S. pneumoniae*. Pneumococci that produce β -lactamase have never been reported so far, and their resistance to penicillins and cephalosporins is entirely attributable to alteration of PBPs. *S. pneumoniae* strains possess five high- M_r PBPs (1A, 1B, 2A, 2B and 2X) and the low- M_r PBP 3, which has not been implicated in the killing action of β -lactam antibiotics. The most highly penicillin-resistant isolates (MIC = 2 – 16 μ g/ml benzyl penicillin) produce altered forms of PBPs 1A, 2X, 2B and 2A that have reduced affinity for β -lactams. Little is known about the biochemical base of the decreased affinity.

The recognition of conserved sequence motifs within the transpeptidase domains of high- M_r PBPs, and in the Serine β -lactamase and low- M_r PBPs, suggest that enzymes have similar structures. The sequence motifs are located around the active

center of the penicillin-interacting enzymes, and as expected the amino acid changes that reduce the affinity of PBP are within, or close to, these conserved sequences.



- 1 = Markers (2, 1.5, 1, 0.75, 0.5, 0.3, 0.15, 0.05 kb)
 2 = *E. faecalis* 15376
 3 = *vanA* positive control BM 4174 strain
 4 = *vanA* negative control ATCC 29212 strain

Fig. 5. Electrophoretic profile of the PCR product with agarose gel electrophoresis after ethidium bromide staining

Features of resistance

The reduction of affinity for different β -lactam drugs varies greatly with the individual compound. The phenomenon of hetero-resistance can easily be demonstrated among pneumococcus isolates. Intermediate PRSPs have MIC = 1–2

$\mu\text{g/ml}$, PRSPs possess MIC = 3–6 $\mu\text{g/ml}$, while high-level PRSPs exhibit a MIC > 6 $\mu\text{g/ml}$. It has also been observed that PRSP strains are often multiple resistant (macrolides, tetracyclines, chloramphenicol, sulfonamide-trimethoprim combination). The incidence of PRSP in Hungary is shown in Table 2.

Genetic basis and phenotype of resistance

The low-affinity forms of the high M_r pneumococcal PBPs have arisen by recombination rather than mutation as seen in pathogenic *Neisseria* spp., too. Pneumococci are naturally transformable, and mosaic structures in their PBP genes is believed to have arisen by interspecies homologous recombination, although it has proven difficult to identify the source, or rather sources of the divergent regions [130]. The altered PBPs have greatly reduced affinity for almost all β -lactam antibiotics, including the 3rd generation cephalosporins. Penicillin-resistant *S. pneumoniae* strains therefore show cross-resistance to other β -lactam antibiotics. In most resistant isolates, the MICs of 3rd generation cephalosporins are equal to, or just slightly less than the MICs of penicillin. The 3rd generation cephalosporins, however, may still be effective in treating pneumococcal infections caused by penicillin-resistant strains because large amount of these antibiotics can be maintained in the blood and cerebrospinal fluid. However, high-level resistance to 3rd generation cephalosporins has recently emerged [131]. In contrast to high-level resistance to penicillin, which requires alteration of four PBPs, clinical pneumococcal isolates with ceftriaxone MIC as high as 16 $\mu\text{g/ml}$ have alteration of only PBPs 1A and 2X, as the other PBPs have inherently low affinity for 3rd generation cephalosporins and therefore are not involved in the killing of pneumococci by clinically relevant concentration of the compounds [132]. Most recently, strains of *S. pneumoniae* with high level penicillin and cefotaxime resistance were isolated from the respiratory tract of in- and outpatients in Hungary [133].

Detection of β -lactam resistance

β -lactam resistance can routinely be screened by 1 μg oxacillin disc (NCCLS). Susceptibility to oxacillin implies susceptibility to all the other β -lactam drugs. However, resistance to oxacillin indicates the need for the determination of penicillin MIC. Penicillin MIC is measured either by penicillin-G E-test, or by one of the dilution (micro- or macro-) methods with Mueller-Hinton broth containing 5% of horse blood as recommended by NCCLS [37]. Recently, polymerase chain reaction (PCR) techniques have also been introduced into the detection of PRSP strains. Using primers derived from the pneumolysin, autolysin, and DNA polymerase I genes, and the 16S – 23S spacer ribosomal region, PCR has been used successfully to show the presence of *S. pneumoniae* in blood, sputum, cerebrospinal fluid of patients. The nucleotide

sequences of genes coding for the native and altered PBPs (2B, 2X and 1A) of both PRSP and penicillin susceptible *S. pneumoniae* have been reported. PCR primers were designed to amplify the differential nucleotide sequences of these genes in penicillin susceptible and resistant strains. The use of PCR to differentiate between penicillin susceptible and resistant genotypes has been established [134].

Glycopeptide resistant *S. pneumoniae* has not been reported yet.

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THE GLUTATHIONE METABOLISM OF THE β -LACTAM PRODUCER FILAMENTOUS FUNGUS *PENICILLIUM CHRYSOGENUM**

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Glutathione (γ -L-glutamyl-L-cysteinyl-glycine; GSH) shares structural similarities with the β -lactam biosynthetic intermediate ACV-tripeptide $\{\delta$ -(L- α -aminoadipyl)-L-cysteinyl-D-valine}. Not surprisingly, GSH has been reported to inhibit the β -lactam biosynthetic machinery quite effectively and, hence, strategies to decrease the intracellular GSH concentrations without influencing negatively the physiological status of idiophasic mycelia would attract industrial interests. Here we present a detailed map of the GSH metabolic network of *P. chrysogenum* and show a promising way to keep the GSH pool selectively down under penicillin producing conditions. This procedure includes a well-controlled and transient lowering of pH at the beginning of the production phase, and it relies on the GSH-dependent detoxification of the protonophore penicillin side-chain precursors phenoxyacetic acid (POA) and phenylacetic acid (PA). Encouraging preliminary fed-batch fermentation experiments have been performed to test this technological proposal. Interestingly, the mechanism of the activation of POA and PA to the appropriate CoA derivatives has remained yet to be answered but the involvement of GSH seems to be rather unlikely in this case. Our data also challenge the hypothesis that the formation of different kinds of penicillins would be an alternative to GSH-dependent detoxification processes in *P. chrysogenum*.

Keywords: *Penicillium chrysogenum*, glutathione metabolism

* This paper was written to commemorate to the fiftieth anniversary of the foundation of the Hungarian Society for Microbiology.

Introduction

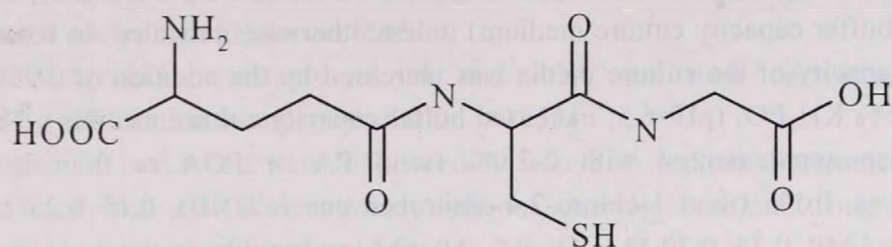
Glutathione (γ -L-glutamyl-L-cysteinyl-glycine; Fig. 1, GSH) is one of the most abundant low molecular weight thiols found in biological systems [1]. In fungi GSH was proved to play a crucial role in numerous physiological processes including (i) maintenance of a suitable reduced milieu for biosynthetic enzymes *via* stabilization of -SH groups [2], (ii) elimination of reactive oxygen species (ROS), e.g. peroxides [3–6], (iii) detoxification of harmful metabolites (e.g. formaldehyde and methylglyoxal) [7, 8], xenobiotics (*via* GSH S-conjugation) [9, 10] and heavy metal ions (e.g. Cd^{2+} and CrO_4^{2-}) [11, 12] as well as (iv) transportation of amino acids and peptides *via* the γ -glutamyl cycle [13]. In addition, GSH may also serve as a major sulphur storage compound in fungi [14].

In terms of cell morphology and cell physiology, GSH/GSSG redox imbalances of fungal cells are considered as early signal transduction events of dimorphic (yeast $\leftrightarrow$ hypha) transitions [15–19], cell differentiation processes including sporulation and germination *via* transient hyperoxidant states (Dioxygen Avoidance Theory of Cell Differentiation) [20–22] and autolysis. It is notable that fungal autolysis more recently turned out to be energy-dependent and, at least in some aspects, reminiscent of the apoptosis of higher eukaryotes [23, 24].

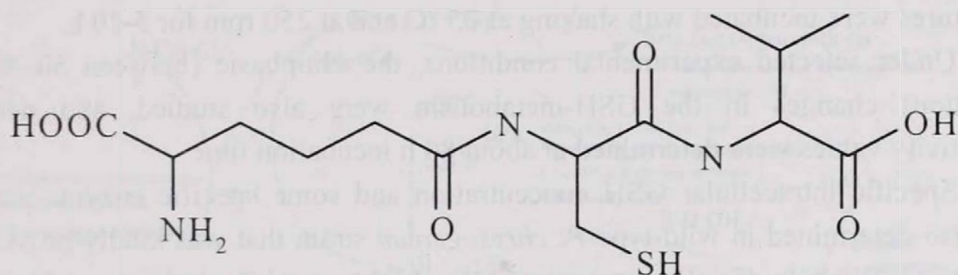
In β -lactam producer filamentous fungi (e.g. *Penicillium chrysogenum*, *Acremonium chrysogenum*) GSH also effects secondary metabolism because one of the most important intermediates of the β -lactam biosynthesis, δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine (ACV), is structurally analogous to GSH (Fig. 1). As a result, GSH suppresses β -lactam production by inhibiting ACV synthase and isopenicillin N synthase (IPNS) activities [25–29]. Paradoxically, the same tripeptide may contribute to the maintenance of the redox stability of the O_2 -consuming IPNS [30, 31]. GSH may also take part in some additional steps of β -lactam biosynthesis including the intracellular transport of precursor amino acids [32, 33] and the activation of the side-chain precursors phenylacetic and phenoxyacetic acids (PA and POA, respectively) *via* the temporary formation of (S-acyl)glutathione intermediates [34]. Under β -lactam producing conditions, intracellular GSH reserves may serve as an endogenous sulphur source as well [35]. To sum up, GSH seems to be one of the most important, small molecular mass regulators of the β -lactam biosynthetic machinery.

The primary aim of mapping the GSH metabolism of *P. chrysogenum* was to develop a new strategy to decrease the intracellular GSH concentrations without influencing negatively the physiological status of idiophasic mycelia and to record the effect on penicillin productivity. Additional goals were to test the hypotheses that GSH is involved in the activation of penicillin side-precursors and the formation of

penicillins is an alternative to GSH-dependent detoxification of the side-chain precursors.



GSH



ACV

Fig. 1. Structural similarities between glutathione and ACV-tripeptide

Materials and methods

Culture conditions; minimal inhibitory concentration (MIC) determinations

The high β -lactam producing strain *P. chrysogenum* NCAIM 00237 was grown in flasks (500 ml) containing 100 ml of complex culture medium containing 2.0% glucose, 0.4% peptone, 0.4% yeast extract, 0.05% MgSO₄, 0.4% K₂HPO₄, 0.2% KH₂PO₄ [36–44]. The mycelia were separated by filtration on sintered glass, were washed and transferred immediately into a defined medium (pH 6.5–7.6) containing 0.4 or 0.93% K₂HPO₄, 0.2 or 0.55% KH₂PO₄, and also supplemented with 0–2.0% glucose, 2.0% 2-deoxy-D-glucose or 2.0% lactose as carbon sources, 0–60 mM sodium L-glutamate (Na-Glu), 0–40 mM (NH₄)₂HPO₄, 40 mM NaNO₃ or 10 mM NaNO₂ as

nitrogen sources and 20–60 mM cysteine (Cys), 20–60 mM methionine (Met) or 0–4.15 mM MgSO_4 as sulphur sources. Culture media normally contained 101 mM (2.0%) glucose, 60 mM (1.0%) Na-Glu and 4.15 mM (0.05%) MgSO_4 as carbon, nitrogen and sulphur sources, respectively, and 0.4% K_2HPO_4 , 0.2% KH_2PO_4 (pH=7.6; normal buffer capacity culture medium) unless otherwise indicated. In some cases the buffer capacity of the culture media was increased by the addition of 0.93% K_2HPO_4 and 0.55% KH_2PO_4 (pH=6.5; increased buffer capacity culture medium). These media were also supplemented with 0–2.0% (w/v) PA or POA or their hydroxylated derivatives, 0.1% (w/v) 1-chloro-2,4-dinitrobenzene (CDNB), 0.15–0.25 % caffeine, 25 mM cAMP, 0.35–0.70 M H_2O_2 , 0.5–2.0 mM *tert*-butyl hydroperoxide (*tert*-BOOH), 50–500 μM menadione (MQ), 0.5 mM diamide (a widely used compound to oxidise intracellular GSH to GSSG instantaneously and stoichiometrically) or 0–300 μM FeCl_3 as required. The starting mycelial dry weight was 5.0 mg/ml in each experiment and all the cultures were incubated with shaking at 25 °C and at 250 rpm for 5–10 h.

Under selected experimental conditions, the idiophasic (between 50–80 h of incubation) changes in the GSH-metabolism were also studied, and penicillin productivity values were determined at about 80 h incubation time.

Specific intracellular GSH concentration and some specific enzyme activities were also determined in wild-type *P. chrysogenum* strain that was kindly provided by Prof. Dr. G. Winkelmann (Department of Microbiology and Biotechnology, University of Tübingen). The wild-type strain was cultured exactly under the same conditions as described above for the industrial strain.

Minimal inhibitory concentration (MIC) values for the penicillin side-chain precursors and their derivatives were determined by agar dilution method using solid media containing 2.0% lactose, 1.0% Na-glutamate, 0.93% K_2HPO_4 , 0.55% KH_2PO_4 , 0.05% MgSO_4 , 2.0% agar (pH 6.5). Approximately 5.0×10^3 *P. chrysogenum* spores were spotted onto agar discs (diameter 3.0 cm, height 1.5 cm) and were incubated at 25 °C for 5 days [42–44].

Enzyme activity determinations and analytical procedures

Specific glutathione producing activity (GPA) [45], and specific glutathione reductase (GR) [46], glutathione peroxidase (GPx) [47], glutathione S-transferase (GST) [48], γ -glutamyltranspeptidase (γ GT) [49], glucose-6-phosphate dehydrogenase (G6PD) [36], catalase [50] and superoxide dismutases (SODs; CuZnSOD and MnSOD) [51] activities were measured in cell-free extracts after disrupting *P. chrysogenum* mycelia by a Type X25 X-press (AB Biox, Göteborg, Sweden).

For monitoring changes in the intracellular concentrations of GSH and oxidized glutathione (GSSG), harvested mycelia were treated with 5% (w/v) 5-sulfosalicylic

acid and the extracts were neutralized with 0.5 M NaOH [40, 42]. The GSH and GSSG concentrations were measured according to Anderson [52].

The formation of β -lactams was quantified by the method of Bundgaard and Ilver [53] using penicillin V potassium salt as standard. Intracellular protein contents were measured according to Peterson [54].

The dissociation constants of PA and POA and their derivatives were determined by pH-titration in aqueous solution [43, 55].

Intracellular iron levels were monitored by atomic absorption spectrometry [44]. Cell-free extracts were also screened for (*S*-phenoxyacetyl)glutathione using a HPLC equipment and a standard prepared according to Ferrero et al. [34].

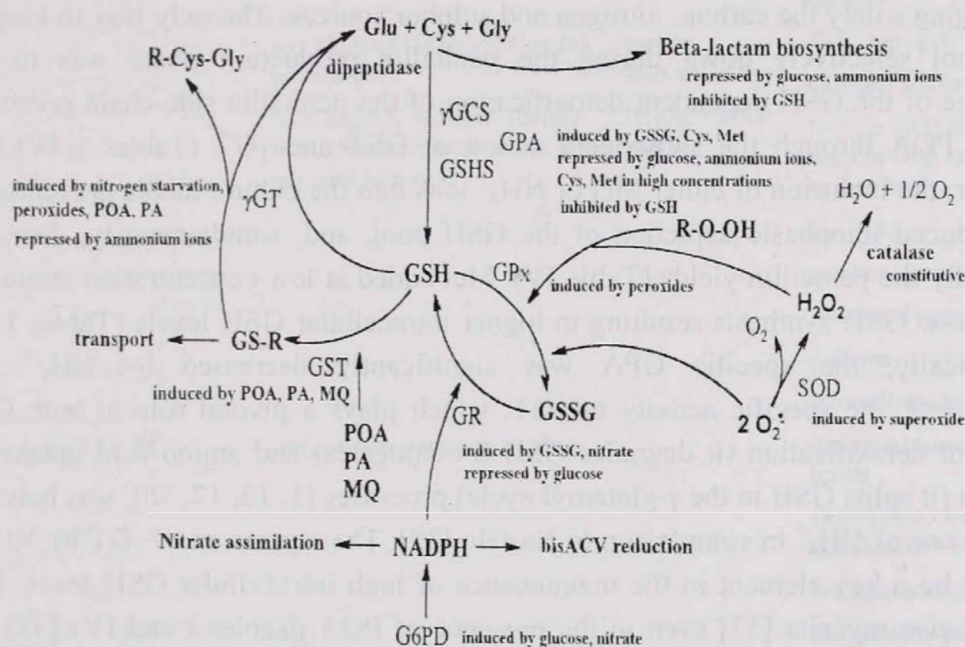


Fig. 2. Summary of the GSH metabolism of *Penicillium chrysogenum* NCAIM 0023

Results and Discussion

*Mapping the GSH-metabolism of a high β -lactam producer industrial strain of *P. chrysogenum**

The complex GSH metabolic network of *P. chrysogenum* NCAIM 0023 is presented in Tables I–III as well as in Fig. 2. Specific penicillin production values observed in normal and increased buffer capacity culture media are summarized in Table IV. Because a more detailed presentation and discussion of experimental data can be found in previous publications [36–44] here we turn our attention only to the most remarkable findings and their interpretation.

One of the most notable observations was that the intracellular GSH levels could not be decreased and, concomitantly, the penicillin yields could not be increased by changing solely the carbon, nitrogen and sulphur sources. The only way to keep the GSH pool selectively down during the penicillin production phase was to take advantage of the GSH-dependent detoxification of the penicillin side-chain precursors PA and POA through the subsequent action of GST and γ GT (Tables I–IV) [43]. However, the inclusion of either Met or NH_4^+ ions into the culture media prevented the POA-induced idiophasic depletion of the GSH pool, and, simultaneously, decreased profoundly the penicillin yields (Table IV). Met added at low concentration stimulated the *de novo* GSH synthesis resulting in higher intracellular GSH levels (Tables I–III). Paradoxically, the specific GPA was significantly decreased by NH_4^+ ions. Nevertheless, the specific activity of γ GT, which plays a pivotal role in both GSH-dependent detoxification (it degrades GSH S-conjugates) and amino acid uptake and transport (it splits GSH in the γ -glutamyl cycle) processes [1, 13, 17, 56], was halved in the presence of NH_4^+ in comparison to Na-Glu [39]. The repression of γ GT by NH_4^+ is likely to be a key element in the maintenance of high intracellular GSH levels in *P. chrysogenum* mycelia [57] even in the presence of POA (Tables I and IV) [38] and, hence, may contribute to the observed ammonium ion repression of the penicillin biosynthesis itself (Table IV) [26]. Fortunately, POA increased the specific activities of the GSH-dependent detoxification pathway even in the presence of NH_4^+ ions (although to a lesser extent as with Na-Glu) [38], which may give us the opportunity to decrease the intracellular GSH pool under penicillin producing conditions by stimulating the influx of side-chain precursors into the cells (see below).

Table I

Specific activities and regulations of GSH-dependent and GSH-metabolism related enzymes in Penicillium chrysogenum^a [36–44]

Enzymes	Specific activity ^b {mkat (kg protein) ⁻¹ }	Specific activity was increased by	Specific activity was decreased by	Specific activity was not affected by
GPA	0.060	any kinds of GSH/GSSG redox imbalances 20 mM Cys or Met carbon starvation lactose carbon source	60 mM Cys or Met NH₄⁺ nitrogen source	nitrogen starvation NO ₃ ⁻ or NO ₂ ⁻ nitrogen sources sulphur starvation 2-deoxy-D-glucose caffeine
GR	3.1	any kinds of GSH/GSSG redox imbalances NO ₃ ⁻ or NO ₂ ⁻ nitrogen sources with glucose carbon source but not with lactose	carbon starvation lactose carbon source	NH ₄ ⁺ nitrogen source nitrogen starvation NO ₃ ⁻ or NO ₂ ⁻ nitrogen sources with lactose carbon source Cys, Met sulphur starvation 2-deoxy-D-glucose caffeine intracellular iron levels
GPx	0.48	intracellular peroxide levels		increased superoxide levels GSH/GSSG redox imbalances nitrogen sources
GST	0.51	MQ penicillin side-chain precursors		intracellular peroxide levels GSH/GSSG redox imbalances nitrogen sources CDNB

Table I (continued)

Enzymes	Specific activity ^b {mkat (kg protein) ⁻¹ }	Specific activity was Increased by	Specific activity was decreased by	Specific activity was not affected by
γGT	0.12	intracellular peroxide levels penicillin side-chain precursors nitrogen starvation	NH₄⁺ nitrogen source	carbon sources sulphur sources intracellular superoxide levels MQ GSH/GSSG redox imbalances CDNB
G6PD	2.4	NO ₃ ⁻ or NO ₂ ⁻ nitrogen sources with glucose carbon source but not with lactose	lactose carbon source caffeine exogenous cAMP	intracellular peroxide levels intracellular superoxide levels GSH/GSSG redox imbalances
Catalase	5800			any tested culture media components or additives
CuZnSOD^c	46 mU (kg protein) ⁻¹	intracellular superoxide levels		intracellular peroxide levels GSH/GSSG redox imbalances
MnSOD^c	18mU (kg protein) ⁻¹	intracellular superoxide levels		intracellular peroxide levels GSH/GSSG redox imbalances

^a - Specific enzyme activities determined at 5-10 h of incubation are summarized.

^b - Culture media contained 101 mM glucose, 60 mM Na-Glu and 4.15 mM MgSO₄ as carbon, nitrogen and sulphur sources, respectively, in the normal buffer capacity culture medium unless otherwise indicated.

^c - One unit of SOD activity was defined as the amount of enzyme that inhibited the Nitro Blue Tetrazolium oxidation rate of the control by 50 % [51]. In the MnSOD assay, reaction mixtures were also supplemented with 4.0 mM NaCN.

Table II

Specific concentrations of GSH and GSSG in Penicillium chrysogenum^a [36–44]

Parameters	Specific concentrations ^b {mmol (kg protein) ⁻¹ }	Specific concentration was increased by	Specific concentration was decreased by	Specific concentration was not affected by
GSH	26	lactose carbon source carbon starvation Cys, Met diamide (paradoxically)	oxidative stress generated by <i>tert</i> -BOOH and MQ sulphur starvation side-chain precursors depending on the pH control of the media but not with NH₄⁺ ions POA added at MIC, CDNB	nitrogen source nitrogen starvation caffeine 2-deoxy-D-glucose H ₂ O ₂ up to 0.70 M intracellular iron levels
GSSG	2.0	oxidative stress generated by any kinds of stressors side-chain precursors depending on the pH control of the media but not with NH₄⁺ ions POA added at MIC CDNB		carbon source nitrogen source sulphur source caffeine

^a – Specific concentrations determined at 5–10 h of incubation are summarized.^b – Culture media contained 101 mM glucose, 60 mM Na-Glu and 4.15 mM MgSO₄ as carbon, nitrogen and sulphur sources, respectively, in the normal buffer capacity culture medium unless otherwise indicated.

In accordance with previous observations [58], the toxicity of the protonophore side-chain precursors, which are transported across the plasma membrane solely by free diffusion [59,60], was dependent on the pH of the culture medium (Tables I–IV) [38,42], and, most likely, was connected with the formation of toxic epoxide intermediates of hydroxylation reactions on the aromatic rings [42, 43]. The idea that other factors than the protonophoric character of these side-chain precursors were responsible for their cell toxicity was supported by the observation that the fungus

could not tolerate POA at 0.7% (equals to MIC for POA) or higher concentrations even in an increased buffer capacity culture medium (Table IV). On the other hand, *P. chrysogenum* was shown to produce penicillin V quite effectively in a normal buffer capacity culture medium containing 0.4% POA, which was characterized with low intracellular GSH concentrations and very severe GSH/GSSG redox imbalances [38].

Table III

GSH/GSSG redox balances in Penicillium chrysogenum^a [36–44]

Parameter	Concentration ratio ^b	Concentration ratio was increased by	Concentration ratio was decreased by	Concentration ratio was not affected by
GSH / GSSG ratio	13	carbon starvation lactose carbon source Cys, Met	oxidative stress generated by any kinds of stressors side-chain precursors depending on the pH control of the media but not with NH₄⁺ ions POA added at MIC sulphur starvation CDNB	nitrogen source caffeine

^a - Concentration ratios determined at 5–10 h of incubation are summarized.

^b - Culture media contained 101 mM glucose, 60 mM Na-Glu and 4.15 mM MgSO₄ as carbon, nitrogen and sulphur sources, respectively, in the normal buffer capacity culture medium unless otherwise indicated.

Importantly, *P. chrysogenum* tolerated any kinds of oxidative stress exceptionally well owing to the very high constitutive catalase and inducible GPx activities (Table I). The intracellular GSH concentrations and the redox capacity of the GSH/GSSG system observed in the trophophase even seemed to outweigh the needs raised later by the β -lactam production [43].

In addition, thioredoxin-dependent redox enzymes, e.g. peroxidases and broad-specificity disulphide reductases, were likely to play a crucial role in both the antioxidant defence of the cells and the stabilization of the ACV-tripeptide in its reduced form especially in the case of GSH/GSSG redox imbalances [61–63].

To sum up, the only technological possibility to keep the intracellular GSH concentrations low in idiophasic *P. chrysogenum* cultures seems to be a well-controlled and transient lowering of pH at the beginning of the production phase. The GSH-dependent elimination of protonophore side-chain precursor molecules flooded into the cells is expected to diminish intracellular GSH concentrations [43].

Table IV

Penicillin yields under different culture conditions [38,42,43]

Culture conditions	Idiophasic GSH levels ^a {mmol (kg protein) ⁻¹ }	Idiophasic GSSG levels ^a {mmol (kg protein) ⁻¹ }	Penicillin yields ^b {mol (kg protein) ⁻¹ }
Increased buffer capacity culture media			
2.0% lactose, 1.0% Na-Glu , 0.93% K ₂ HPO ₄ , 0.55% KH ₂ PO ₄ , 0.05% MgSO ₄ , pH=6.5	60–65	1.1–1.4	0.1
2.0% lactose, 1.0% Na-Glu , 0.93% K ₂ HPO ₄ , 0.55% KH ₂ PO ₄ , 0.05 %MgSO ₄ , pH=6.5, 0.5% POA	12	4.0	3.5
2.0% lactose, 1.0% Na-Glu , 0.93% K ₂ HPO ₄ , 0.55% KH ₂ PO ₄ , 0.05% MgSO ₄ , pH=6.5 0.5% POA , +40 mM Met at 35 h of incubation	20–25	not determined	1.5
2.0% lactose, 1.0% Na-Glu , 0.93% K ₂ HPO ₄ , 0.55% KH ₂ PO ₄ , 0.05% MgSO ₄ , pH=6.5, 0.7% POA (MIC)	not detectable starting from 15 h of incubation	44 at 25 h of incubation because of serious oxidative cell injuries	not determined
Normal buffer capacity culture media			
2.0% lactose, 0.8% Na-Glu , 0.40% K ₂ HPO ₄ , 0.20% KH ₂ PO ₄ , 0.05% MgSO ₄ , pH=7.6,	85 at 15 h of incubation	2.3 at 15 h of incubation	0.1

Table IV (continued)

Culture conditions	Idiophasic GSH levels ^a {mmol (kg protein) ⁻¹ }	Idiophasic GSSG levels ^a {mmol (kg protein) ⁻¹ }	Penicillin yields ^b {mol (kg protein) ⁻¹ }
Normal buffer capacity culture media			
2.0% lactose, 0.8% Na-Glu , 0.40% K ₂ HPO ₄ , 0.20% KH ₂ PO ₄ , 0.05% MgSO ₄ , pH=7.6, 0.4% POA	not detectable starting from 10 h of incubation	38 at 15 h of incubation	2.0
2.0% lactose, 0.8% Na-Glu , 0.40% K ₂ HPO ₄ , 0.20% KH ₂ PO ₄ , 0.05% MgSO ₄ , pH=7.6, 0.1% CDN	not detectable starting from 10 h of incubation	41 at 15 h of incubation	not detectable
2.0% lactose, 1.5% (NH₄)HPO₄ , 0.40% K ₂ HPO ₄ , 0.20% KH ₂ PO ₄ , 0.05% MgSO ₄ , pH=7.6,	74 at 15 h of incubation	2.1 at 15 h of incubation	not detectable
2.0% lactose, 1.5% (NH₄)HPO₄ , 0.40% K ₂ HPO ₄ , 0.20% KH ₂ PO ₄ , 0.05% MgSO ₄ , pH=7.6, 0.4% POA	81 at 15 h of incubation	2.5 at 15 h of incubation	not detectable

^a – Unless otherwise indicated, specific GSH and GSSG concentrations determined between 50 and 80 h of incubation are shown.

^b – Highest penicillin productivity values observed at about 80 h of incubation are presented.

This proposal has been tested preliminarily in a 5 l fed-batch penicillin V fermentation system [64, 65] using (NH₄)₂SO₄ as a nitrogen source and introducing different degrees of acidification for 5 h at the beginning of the production phase. When the pH was decreased from 6.4 to 5.4 a decrease in the intracellular GSH levels and some increase in the penicillin V yields were recorded (Sámi, L., personal communication). Further fermentation experiments are in progress to optimize the degree of acidification and to minimize its negative physiological effects (e.g. decreasing biomass [42]) under penicillin V producing conditions.

Obviously, the increased influx of toxic side-chain precursor molecules into idiophasic cells gives a certain risk to the fermentation process, which is rather difficult to estimate and control. Therefore, the construction of low GSH producer *P. chrysogenum* strains preferably also defective in the degradation of penicillin side-chain precursors can be highly recommended as a new strategy for further *P. chrysogenum* strain improvement [66, 67].

Table V

*Specific glucose-6-phosphate dehydrogenase and catalase activities and specific GSH productions in a high β -lactam producer and in a wild-type strain of *Penicillium chrysogenum*^a*

Strain	G6PD ^b mkat (kg protein) ⁻¹	catalase ^b kat (kg protein) ⁻¹	GSH ^b mmol (kg protein) ⁻¹
NCAIM 00237	2.4±0.2 ^c	5.4±0.7 ^c	25±2
Wild-type	2.3±0.2 ^c	4.2±0.6 ^c	23±2

^a – Culture media contained 101 mM glucose, 60 mM Na-Glu and 4.15 mM MgSO₄ as carbon, nitrogen and sulphur sources, respectively, in the normal buffer capacity culture medium unless otherwise indicated.

^b – Specific enzyme activity and specific GSH production values are expressed as Mean±S.D., calculated from 4 independent experiments.

^c – Specific G6PD and catalase activities were not inducible by 0.35 M H₂O₂ [37].

Table VI

Ranking of acidity, toxicity, GSH-dependent detoxification and penicillin productivity of PA, POA and their hydroxylated derivatives [43]

Acidity (pK_i):

2-hydroxy-POA>POA≅4-hydroxy-POA>>PA≅3-hydroxy-PA≅2,5-dihydroxy-PA>4-hydroxy-PA≅2-hydroxy-PA>3,4-dihydroxy-benzoic acid

Toxicity (MIC):

PA>POA≅2-hydroxy-PA>2-hydroxy-POA>3-hydroxy-PA≅4-hydroxy-PA>4-hydroxy-POA≅2,5-dihydroxy-PA≅3,4-dihydroxy-benzoic acid

GSH-dependent detoxification (induction of GST and γ GT):

PA>POA>2-hydroxy-PA≅2-hydroxy-POA>3-hydroxy-PA>4-hydroxy-PA≅4-hydroxy-POA≅2,5-dihydroxy-PA≅3,4-dihydroxy-benzoic acid

β -Lactam production:

PA>POA>>4-hydroxy-PA≅4-hydroxy-POA>3-hydroxy-PA>2-hydroxy-PA≅2-hydroxy-POA≅2,5-dihydroxy-PA≅3,4-dihydroxy-benzoic acid

*Comparison of catalase and glucose-6-phosphate dehydrogenase activities in a high β -lactam producer and in a wild-type *P. chrysogenum* strain*

As shown previously (Table I), high specific catalase and G6PD activities were found in *P. chrysogenum* NCAIM 0023 mycelia that were insensitive to either intracellular ROS concentrations or GSH/GSSG redox imbalances. The lack of regulation of these enzyme activities by oxidative stress is very unusual among fungi [3, 68]. G6PD is a key enzyme of the pentose phosphate pathway that is one of the major NADPH suppliers of *P. chrysogenum* cells. It is wellknown that the biosynthesis of the penicillin precursor amino acids L- α -aminoadipic acid, L-cysteine and L-valine requires at least 8 NADPH [69, 70], and the stabilization of the ACV-tripeptide in its reduced form is also likely to need significant quantities of NADPH [61, 62]. Therefore, the presence or absence of any regulatory element of the pentose phosphate pathway in *P. chrysogenum* may have an effect on the β -lactam production as well. Similarly, catalase may be of crucial importance in the elimination of peroxide generated by glucose oxidase [71]. According to Nielsen [70], when *P. chrysogenum* was grown in a defined medium, the yield of gluconate was as high as 0.60 moles gluconate (and, of course, 0.60 moles H_2O_2 also formed) per mole glucose. Obviously, an effective antioxidative enzyme system is needed to cope with the burst of ROS under these conditions.

As indicated in Table V, essentially the same specific G6PD and catalase activities were detected both in the high β -lactam producer *P. chrysogenum* NCAIM 00237 strain and in a wild-type strain. This means that the high G6PD and catalase activities not inducible by oxidative stress are intrinsic features of *P. chrysogenum* and are not the consequences of strain development. Obviously, these metabolic characteristics contributed to the success of *P. chrysogenum* as an industrial micro-organism.

As far as other antioxidative enzymes are concerned, more recently Diez et al. [72] did not find any correlation between MnSOD gene expression levels and penicillin G productivity in different *P. chrysogenum* strains.

It is worth noting that the strain improvement procedures did not influence the specific intracellular GSH concentrations either in *P. chrysogenum* NCAIM 00237 (Table V).

The activation of penicillin side-chain precursors

Interestingly, there are still some uncertainties in connection with the formation of CoA-activated PA and POA derivatives in idiophasic *P. chrysogenum* mycelia [29]. There are three options to form CoA-PA and CoA-POA in *P. chrysogenum* cells

including cytosolic acetyl-CoA synthase [73], microsomal phenylacetyl-CoA ligase [29, 74] or GST through the temporary formation of GSH S-conjugates of PA and POA [34]. Although S-conjugated PA was reported to be a good substrate for the microbody-located acyl-CoA:isopenicillin N acyltransferase [34] we failed to detect any (S-phenoxyacetyl)glutathione intermediate in idiophasic *P. chrysogenum* cells under penicillin V production conditions [42], which makes this route rather unlikely to function *in vivo*. Further research is needed to decide if either one of or both acetyl-CoA synthase and phenylacetyl-CoA ligase are involved in penicillin biosynthesis *in vivo* [29].

Is β -lactam production a detoxification process in P. chrysogenum? Is it an alternative to GSH-dependent detoxification?

Unfortunately, our data presented in Table VI do not support this exiting hypothesis. The toxicity of penicillin side-chain precursors and their hydroxylated derivatives correlated well with GSH-dependent detoxification but there was no correlation with antibiotic production. As it is certainly the case for many fungal antibiotics [75], β -lactams may play an important role in the defence of territory and nutrients of some *Penicillium* species [76].

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VERTICAL TRANSMISSION OF HUMAN IMMUNODEFICIENCY VIRUS

(A REVIEW)*

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Sensitive detection methods, such as DNA PCR and RNA PCR suggest that vertical transmission of human immunodeficiency virus (HIV) occurs at three major time periods; *in utero*, around the time of birth, and postpartum as a result of breastfeeding (Fig. 1). Detection of proviral DNA in infant's blood at birth suggests that transmission occurred prior to delivery. A working definition for time of infection is that HIV detection by DNA PCR in the first 48 h of life indicates *in utero* transmission, while peripartum transmission is considered if DNA PCR is negative the first 48 h, but then it is positive 7 or more days later [1]. Generally, in the breastfeeding population, breast milk transmission is thought to occur if virus is not detected by PCR at 3–5 months of life but is detected thereafter within the breastfeeding period [2]. Using these definitions and guidelines, studies have suggested that in developed countries the majority, or two thirds of vertical transmission occur peripartum, and one-third *in utero* [3–6]. The low rate of breastfeeding transmission is due to the practice of advising known HIV-positive mothers not to feed breast milk. However, since the implementation of antiretroviral treatment in prophylaxis of HIV-positive mothers, some studies have suggested that *in utero* infection accounts for a larger percentage of vertical transmissions [7]. In developing countries, although the majority of infections occurs also peripartum, a significant percentage, 10–17%, is thought to be due to breastfeeding [2, 8, 9].

Keywords: HIV, vertical transmission, routes of infection

* This paper was written to commemorate to the fiftieth anniversary of the foundation of the Hungarian Society for Microbiology.

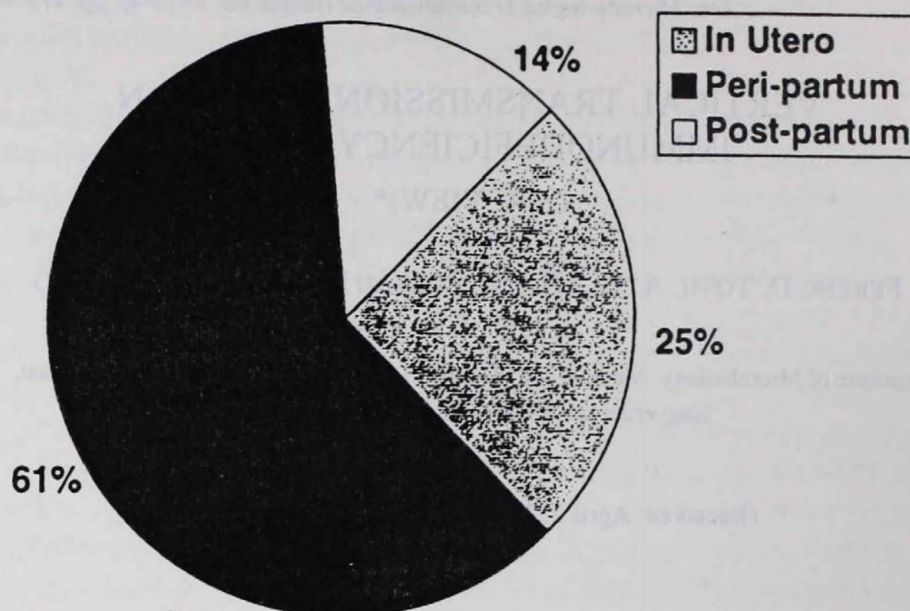


Fig. 1. Timing of vertical HIV infection: estimate of percent attributable to *in utero*, peripartum and postpartum (breast feeding) transmission

Transplacental transmission of HIV

Syncytiotrophoblast forms a continuous, multinucleated epithelium (Fig. 2) that must be traversed by HIV in order to reach underlying cytotrophoblast cells, placental macrophages, fibroblasts, and endothelial cells lining the fetal capillaries. Transmission through the placenta could involve (I) direct transmission of infected cells (monocytes or T cells) from the mother through lesions of the placental barrier, (II) transcytosis, i.e. transport across the trophoblast layer of infected cells, (III) infection of trophoblasts, (IV) entry of the virus in the form of virus/maternal antibody complex.

HIV infection of syncytiotrophoblast

Direct evidence of the infection of syncytiotrophoblast was described. Using immunocytochemical and *in situ* hybridization techniques, researchers detected HIV antigens and genetic material in trophoblast samples obtained from HIV-infected women [10–13].

The problem of the infection of syncytiotrophoblast cells remains controversial. Even if HIV has been detected in the syncytiotrophoblast layer *in situ* [10–12], *in vitro* studies are more conflicting. Several studies demonstrated that cultured human

trophoblasts are moderately, but effectively, susceptible to infection by laboratory strains of HIV [14–16]. In contrast, other investigators have been unable to demonstrate any trophoblast infection with cell-free virus [17, 18] and claim that cell-cell contact is an absolute requirement for HIV infection of syncytiotrophoblast cells.

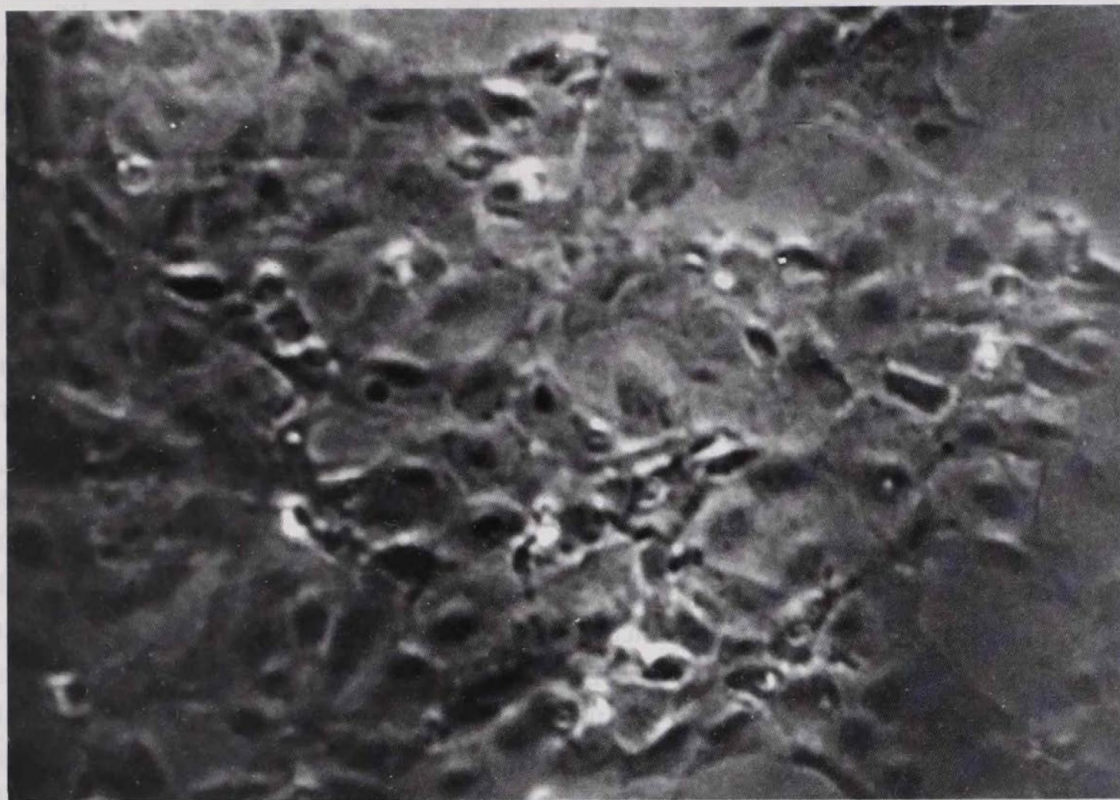


Fig. 2. Human syncytiotrophoblast in culture

The conflicting results may be due to several experimental variables, such as differences in HIV isolates used for infection of syncytiotrophoblast cells or the use of different detection assays. Fazely et al. [19] demonstrated that placental syncytiotrophoblast cells can be infected with cell-free HIV, but cell-associated infection of syncytiotrophoblasts is more efficient than the infection with cell-free virus. Viral particles were observed in coated pits at the syncytiotrophoblast cell surface. This observation is consistent with an endocytosis-mediated mechanism of virus entry. Results from a recent study [20] suggest that cell-free HIV can enter syncytiotrophoblasts and the susceptibility of these cells to penetration by the virus is strain dependent. Furthermore, infectious HIV strains do not require cell surface CD4

or chemokine receptors to gain entry into syncytiotrophoblast cells. Other reports of the infection of human syncytiotrophoblast culture with cell-free HIV indicate that, at best, only low level of productive viral replication is achieved [14, 15]. The lack of virus production in syncytiotrophoblast cells after *in vitro* contact with cell-free HIV indicate post entry restriction of replication. It is conceivable, however, that productive infection of trophoblast *in vivo* might occur in defined, transient conditions. Observations in support of this assumption include detection of HIV p24 antigen in the placental trophoblastic cells of HIV-positive gravidae [10,12]. These findings suggest that multiple cofactors are likely to be involved in the transplacental transmission of the virus. The stimulatory effect of tumor necrosis factor- α on HIV gene expression in syncytiotrophoblast cells has been demonstrated [21]. Another possible mechanism for the stimulation of HIV replication may be co-infection with other viruses. A good candidate for this scenario is human cytomegalovirus [16]. The syncytiotrophoblasts as an overlapping cell population can be coinfecting with HIV and human cytomegalovirus, and HIV replication is markedly upregulated by previous or simultaneous infection of the cells with cytomegalovirus (Fig. 3).

The phenomenon of antibody-dependent enhancement of virus infection is another means by which HIV can enter cells. In this scheme, virus is internalized as an immune complex via interaction with cell surface Fc receptors or with complement receptors. Antibody-dependent enhancement of HIV binding and infection of certain cells were demonstrated *in vitro* [22, 23]. Human syncytiotrophoblast plasma membrane expresses Fc receptors, and these receptors may function in maternal-fetal transmission of immunoglobulin [24]. Immunohistochemical studies suggest that syncytiotrophoblast has low numbers of CR2-like complement receptors [25, 26]. HIV infection of human trophoblast cells in the presence of HIV antibody-positive serum was first reported by David et al. [27]. In an other study [28] it has been shown that both Fc receptor-mediated and complement-mediated antibody-dependent enhancement can contribute to the uptake of HIV/antibody complexes by syncytiotrophoblast cells and that Fc receptor-mediated antibody-dependent enhancement is more efficient than complement-mediated antibody-dependent enhancement (Figs 4 and 5).

Anchoring villi and chorioamnion

While it is generally assumed that maternal-fetal transmission of HIV would be achieved across the syncytiotrophoblast of free placental villi, two other possible routes of infection warrant consideration.

One possible route involves the anchoring villi. As the placenta develops, specialized structures known as anchoring villi and cell columns are formed at the junction of fetal and maternal tissue [29]. Their unusual organization affords a potential

pathway for viral entry into fetal tissues that does not involve passage of virus across syncytiotrophoblast. HIV could pass from uterine cells and stroma, go between cytotrophoblastic cells of the cell columns, and enter fetal stroma. The cytotrophoblastic cells are joined by desmosomes but lack tight junctions.

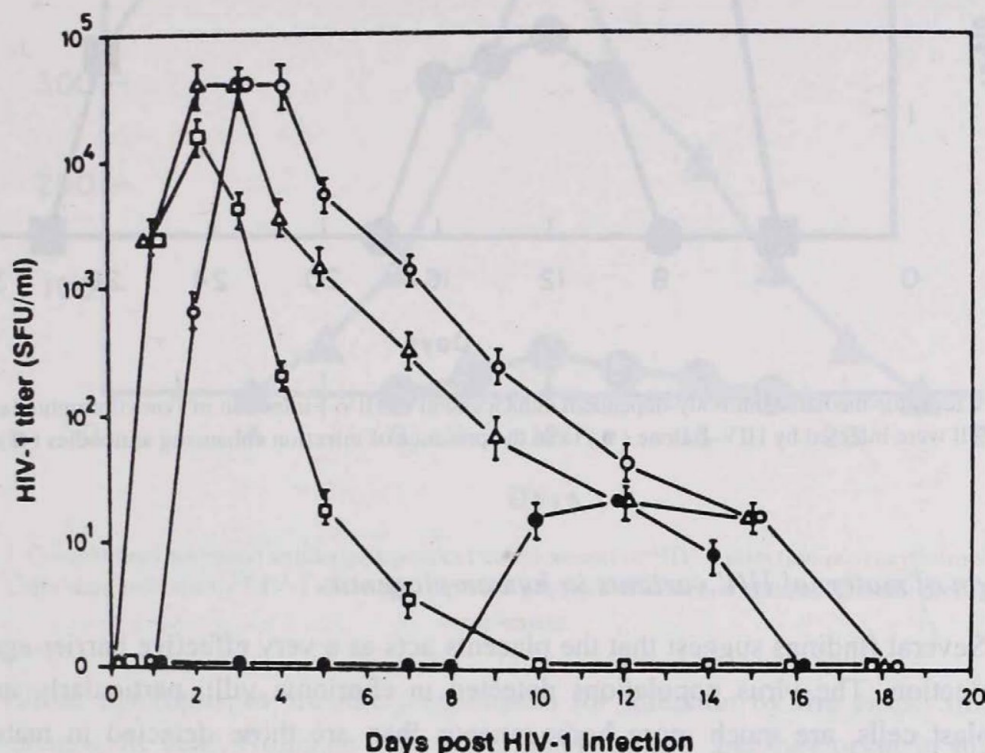


Fig. 3. Effect of HCMV infection on HIV-1 production. Syncytiotrophoblast cells were infected with HIV-1 alone (●), HCMV and HIV-1 simultaneously (○), HCMV for 1 day and then HIV-1 (Δ), or HCMV for 4 days and then HIV-1 (□). The amount of HIV-1 was determined by syncytium-forming assay

Another potential route of HIV infection of the fetus is across the chorioamnion. Here, in late gestation cellular trophoblast abuts on maternal decidua. The decidua contains maternal blood vessels, macrophages and lymphocytes and thus may serve to expose the adjacent trophoblast to HIV. From here, virus or cells may cross the fetal connective tissue and amnion and enter the amniotic fluid. HIV has been isolated from amniotic fluid and cells, but the pathway by which virus enters the fluid is unknown [30].

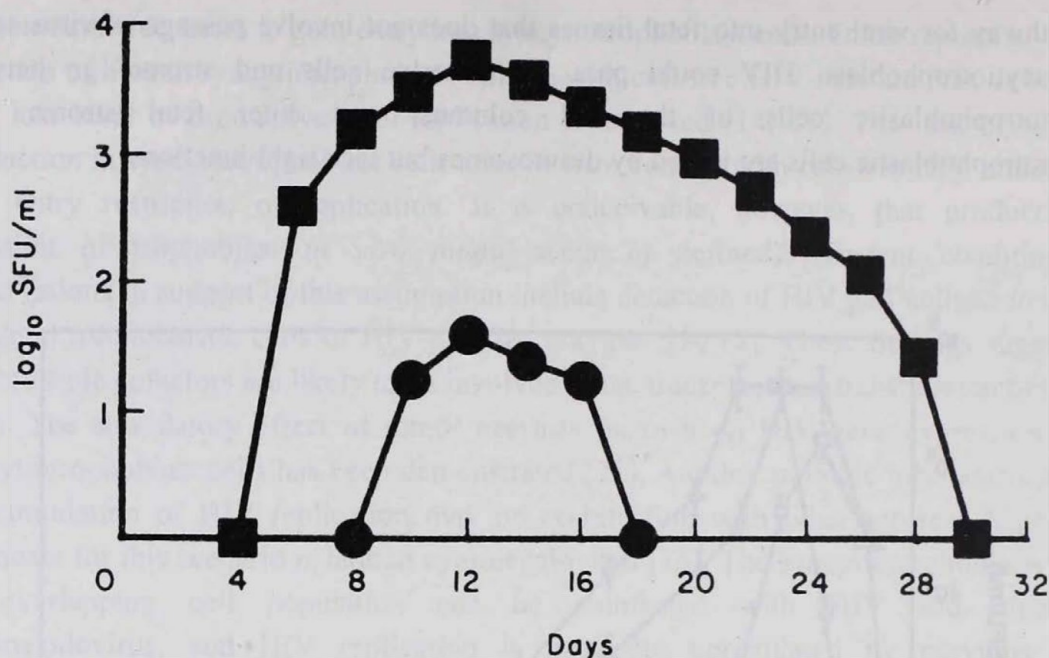


Fig. 4. Fc receptor-mediated antibody-dependent enhancement of HIV-1 infection in syncytiotrophoblast cells. Cells were infected by HIV-1 alone (●) or in the presence of infection enhancing antibodies (■)

Selection of maternal HIV variants in human placenta

Several findings suggest that the placenta acts as a very effective barrier against HIV infection. The virus populations detected in chorionic villi, particularly in the trophoblast cells, are much more homogeneous than are those detected in maternal peripheral blood mononuclear cells throughout the pregnancy [31, 32]. These data indicate that trophoblastic cells may also be involved in the selective process. Placental cell populations other than trophoblastic cells may also be involved in selection of HIV within the placenta. According to some reports, viruses with macrophage tropism are transmitted preferentially during pregnancy [33, 34].

The role of Hofbauer cells, macrophages believed to be of fetal origin and present in the mesenchymal core of the chorionic villi [35], is also of interest in viral pathogenesis. They are present in the villi prior to the development of bone marrow. They maintain their ability to undergo mitosis and self-replicate, in contrast to bone marrow-derived macrophages. *In situ* PCR revealed the presence of HIV proviral DNA in Hofbauer cells in placentas of HIV-infected mothers [10, 36]. Hofbauer cells support infection and replication of HIV and do not show the cytopathic effect of infection even when measurable infection has occurred [37]. Transport of HIV across the syncytiotrophoblast barrier would be particularly efficient in delivery of the virus to its

first target cells, since placental macrophages lay immediately under the trophoblast layer [38].

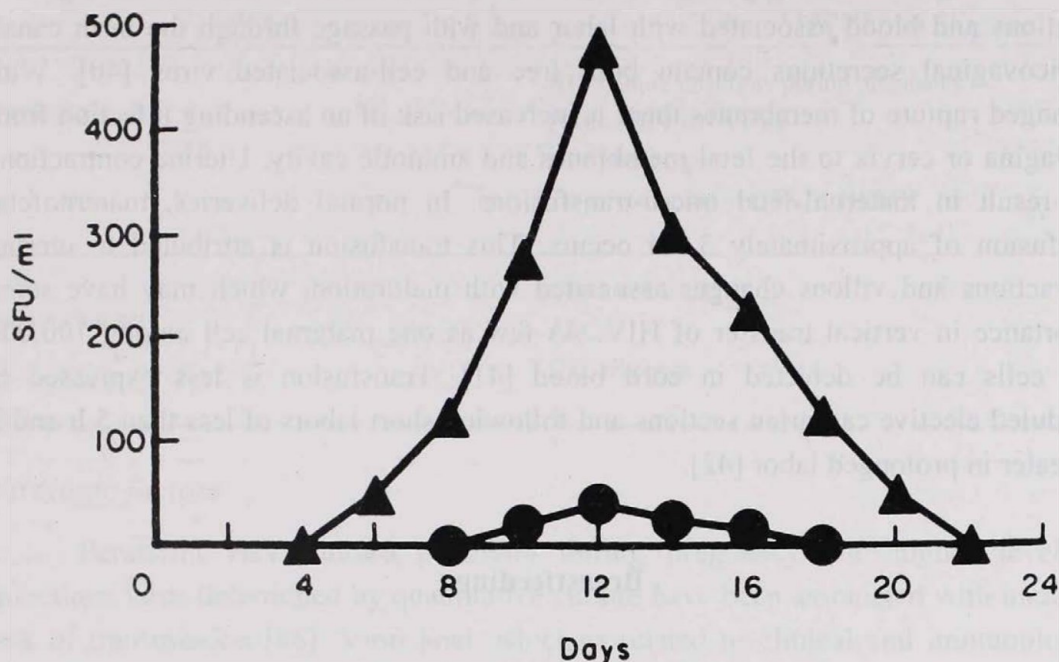


Fig. 5. Complement-mediated antibody-dependent enhancement of HIV-1 infection in syncytiotrophoblast cells. Cells were infected by HIV-1 alone (●) or in the presence of infection enhancing antibodies (▲) plus complement

The villous macrophages are likely candidates for infection by the virus. Similar to other phagocytic cells, Hofbauer cells are probably motile, and they occur in sufficient numbers to permit the transfer of HIV to other cells. Endothelial cells, lining placental blood vessels may also become infected with the virus.

It is conceivable that the spread and selection of maternal virus variants within the placenta results from a cascade of infection of distinct placental cell populations. Altogether, maternal HIV variants appear to undergo a strong negative selection by different cell populations within the placental villi. This may explain why the transplacental transmission of HIV is less frequent than it would be anticipated by the detection of proviral DNA in 70% of placentas from HIV-infected women [6, 39].

Intrapartum infection

During labor and vaginal delivery there is direct contact with maternal genital secretions and blood associated with labor and with passage through the birth canal. Cervicovaginal secretions contain both free and cell-associated virus [40]. With prolonged rupture of membranes there is increased risk of an ascending infection from the vagina or cervix to the fetal membranes and amniotic cavity. Uterine contractions also result in maternal-fetal micro-transfusions. In normal deliveries, maternofetal transfusion of approximately 3 ml occurs. This transfusion is attributed to uterine contractions and villous changes associated with maturation, which may have some importance in vertical transfer of HIV. As few as one maternal cell among 100,000 fetal cells can be detected in cord blood [41]. Transfusion is less expressed in scheduled elective caesarian sections and following short labors of less than 5 h and it is greater in prolonged labor [42].

Breastfeeding

Breastfeeding is an important HIV risk factor, particularly in developing countries. Colostrum and breast milk have been shown to contain virus [43]. Infant infection through breastfeeding is thought to occur in 14% of chronically infected women and in 29% with acute infection [8]. Increasing viral load (by DNA PCR) with time has been detected in breast milk postpartum [44]. This is consistent with the findings that the risk for breast milk transmission appears to increase with duration of breastfeeding [8, 45]. Furthermore, the increased risk seems to be significant 4-6 months postpartum, suggesting that if these findings are verified it may be appropriate to recommend that in some developing countries HIV-positive mothers could breast feed infants until 3 months of age.

Factors associated with transmission

Multiple maternal characteristics have been associated with increased risk of mother-to-child transmission. These characteristics can be loosely categorized as virologic and immunological cofactors (Table I).

Table I

Factors associated with vertical transmission of HIV

Category	Factors
Virologic	HIV culture positivity during pregnancy
	Plasma HIV RNA load
	HIV phenotype
	Co-infecting viruses: HCV, HCMV
Immunological	CD4+ cell count
	ADCC
	CTL response

Virologic factors

Persistent HIV culture positivity during pregnancy and higher levels of infectious virus determined by quantitative culture have been associated with increased risk of transmission [46]. Viral load, which is related to clinical and immunological status in the mother, is the main contributing factor for HIV vertical transmission. Higher maternal plasma HIV RNA level has been associated with increased risk, but a threshold by which transmission will likely occur has not been defined consistently and clearly [47, 48]. Although both T-cell-tropic and macrophage-tropic viral sequences can be found in the placental tissue [49], viruses with macrophage tropism are transmitted preferentially during pregnancy [33, 34]. These data indicate that Hofbauer cells may be involved in selection of maternal HIV variants within the placenta.

Associated viral infections could also act as cofactors. Early studies in Italy showed that women infected with hepatitis C virus and HIV had a higher transmission rate [50]. Increased HIV vertical transmission associated with maternal hepatitis C virus infection has also been demonstrated in a recent study [51]. Maternal immune status has a major effect on the incidence of congenital infections. Prior maternal immunity to human cytomegalovirus protects the fetus from intrauterine infection. HIV-infected women are likely to be at high risk to transmit cytomegalovirus to their infants, regardless of whether they have primary or recurrent cytomegalovirus infection during pregnancy. Women with both HIV and cytomegalovirus infection may have decreased immune function and increased viraemia when compared to women infected with HIV alone. The incidence of cytomegalovirus infection in congenitally HIV-infected children is 61%, whereas it is only 8% in HIV seroconverters [52]. It has been shown that congenital cytomegalovirus infection is more common in HIV-infected

infants than in HIV-uninfected infants [53]. Analysis of the patterns of HIV and cytomegalovirus replication in singly and dually infected syncytiotrophoblast cells has shown that replication of HIV is markedly upregulated by previous or simultaneous infection of the cells with cytomegalovirus. On the other hand, prior HIV infection of the cells converts cytomegalovirus infection from a nonpermissive state to a permissive one. These data suggest that interactions between HIV and cytomegalovirus in coinfecting syncytiotrophoblast cells may contribute to the transplacental transmission of both viruses [16]. Another potential mechanism for increased cytomegalovirus transmission in HIV-infected pregnant women would result from increased recruitment of HIV-infected macrophages in the presence of cytomegalovirus endocervicitis [54]. The presence of these macrophages could increase the risk of perinatal transmission. Infection with cytomegalovirus in HIV-positive children is an unfavorable prognostic factor for the outcome of HIV disease [55, 56].

Immunological factors

The immunological status of the HIV-infected mothers has also been evaluated. Low maternal CD4+ lymphocyte counts has been linked to higher transmission risk in several studies [57, 58]. Recent studies also demonstrated the importance of activated CD8 cell markers as a sign of viraemia in transmission and their similar interaction with positive culture status [46, 59].

In the infant, although theoretically antibody-dependent cellular cytotoxicity (ADCC) activity could be an important defense against HIV infection, in a limited mother-infant study, generation of ADCC was shown to be delayed in newborns and was not associated with decreased risk [60]. However, HIV-specific cytotoxic T lymphocytes (CTL) have been detected in uninfected infants born to HIV-positive mothers, suggesting that an appropriate host CTL response could be protective against infection [61].

Conclusions

Despite the changing epidemiology of HIV vertical transmission in developed countries due to the relative acceptance and success of maternal and infant antiretroviral therapy as prophylaxis, mother-to-infant transmission continues to cause unacceptable morbidity and mortality in both developing and developed countries. To further decrease transmission rates worldwide, a continuing challenge is necessary to better understand the pathogenesis of HIV vertical transmission.

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THE BIOCHEMISTRY OF CITRIC ACID ACCUMULATION BY *ASPERGILLUS NIGER* (A REVIEW)*

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Fungi, in particular *Aspergilli*, are well known for their potential to overproduce a variety of organic acids. These microorganisms have an intrinsic ability to accumulate these substances and it is generally believed that this provides the fungi with an ecological advantage, since they grow rather well at pH 3 to 5, while some species even tolerate pH values as low as 1.5. Organic acid production can be stimulated and in a number of cases conditions have been found that result in almost quantitative conversion of carbon substrate into acid. This is exploited in large-scale production of a number of organic acids like citric-, gluconic- and itaconic acid. Both in production volume as well as in knowledge available, citrate is by far the major organic acid.

Citric acid (2-hydroxy-propane-1,2,3-tricarboxylic acid) is a true bulk product with an estimated global production of over 900 thousand tons in the year 2000. Till the beginning of the 20th century, it was exclusively extracted from lemons. Since the global market was dominated by an Italian cartel, other means of production were sought. Chemical synthesis was possible, but not suitable due to expensive raw materials and a complicated process with low yield. The discovery of citrate accumulation by *Aspergillus niger* led to a rapid development of a fermentation process, which only a decade later accounted for a large part of the global production.

The application of citric acid is based on three of its properties: (1) acidity and buffer capacity, (2) taste and flavour, and (3) chelation of metal ions. Because of its three acid groups with pK_a values of 3.1, 4.7 and 6.4, citrate is able to produce a very low pH in solution, but is also useful as a buffer over a broad range of pH values (2 to 7). Citric acid has a pleasant acid taste which leaves little aftertaste. It sometimes enhances flavour, but is

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also able to mask sweetness, such as the aspartame taste in diet beverages. Chelation of metal ions is a very important property that has led to applications such as antioxidant and preservative. Moreover, it is a "natural" substance and fully biodegradable.

Keywords: citric acid accumulation, *Aspergillus niger*

Metabolism of citric acid biosynthesis

Early studies by Martin & Wilson [1] and Cleland & Johnson [2] showed that in *A. niger*, citric acid is mainly formed via glycolysis with subsequent condensation of a C₄ unit with a C₂ moiety. In short, citric acid biosynthesis involves uptake of the sugar substrate, glycolytic catabolism of glucose to 2 moles of pyruvate, their subsequent conversion to oxaloacetate and acetyl-CoA, condensation of these two presursors to citric acid and finally, excretions of citric acid from mitochondria and from mycelia, respectively (Fig. 1).

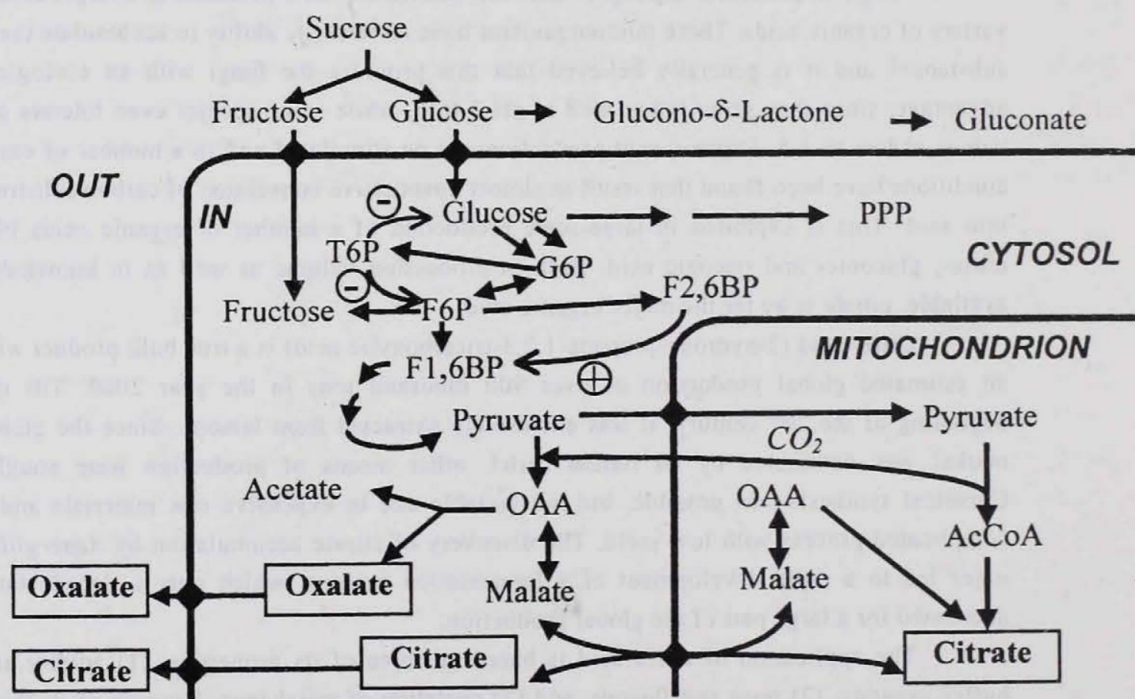


Fig. 1. Overview of pathways leading to citric acid production

Extracellular catabolism of glucose

The possession of glucose-oxidase is common within *A. niger* strains (Mischak et al. [3], Roukas & Harvey [4]). This enzyme is induced by high concentrations of glucose and strong aeration in the presence of low concentrations of other nutrients, conditions which are also typical for citric acid fermentation. Hence, glucose oxidase will inevitably be formed during the starting phase of fermentation and convert a significant amount of glucose into gluconic acid. However, due to the extracellular location of the enzyme, it is directly influenced by the external pH and will be inactivated at $\text{pH} < 3.5$ (Mischak et al. [3], Dronawat et al. [5]). Because of the pK-values for citric acid, pH of the culture filtrate is to decrease to pH 1.8, thereby inactivating glucose-oxidase. It is not known if – and by which mechanism – can gluconic acid be catabolized to citric acid during fermentation.

Sugar uptake

Kinetic analysis Torres et al. [6] shows that *A. niger* possesses at least two glucose transporters. High-affinity glucose transport (K_m 0.3 mM) was detected after growth on low (1%) glucose concentration, whereas an additional low-affinity permease was only found in mycelium cultured in the presence of high (15%) glucose concentrations. The latter may therefore contribute to the increased glycolytic flux during growth on high glucose concentrations. Surprisingly, despite of the fact that the transport of fructose is also relevant for sucrose-based citric acid production, no data is available for *A. niger* in this respect.

Sugar phosphorylation

The first metabolic step after uptake is the phosphorylation of the sugar. Two hexose phosphorylating enzymes exist in *A. niger*, a hexokinase and a glucokinase (Panneman et al. [7, 8]). Both enzymes are present under citric acid producing conditions, but their contribution to the phosphorylation of glucose and fructose is different. The hexokinase is able to phosphorylate both glucose and fructose, whereas glucokinase has a very low, physiologically irrelevant affinity for fructose and a very high (K_m 0.06 mM) affinity for glucose. Hexokinase is strongly inhibited by trehalose 6-phosphate, an intermediate of trehalose biosynthesis (Panneman et al. [8]). By removing this inhibition glycolytic flux could successfully be increased (Arisan-Atac et al. [9]) and by disrupting the gene encoding trehalose 6-phosphate synthase, a higher rate of citric acid production from sucrose in the early phase of accumulation was observed. Since glucokinase is not sensitive for trehalose 6-phosphate inhibition, this approach may not be successful when glucose is the carbon source of choice.

From the level of glucose 6-phosphate, carbon metabolism can either proceed via the glycolytic or the pentose-phosphate pathway. In earlier stages of the fermentation pentose-phosphate pathway dominates and provide the cell with building blocks and NADPH, while later it accounts only for a minor fraction and carbon is mainly metabolized via glycolysis. It was speculated that this may be due to the inhibitory effect of citrate on 6-phosphogluconate dehydrogenase, but evidence for this is lacking. It should be noted that both arabitol and erythritol are accumulated as byproducts until late stages of fermentation (Röhr et al. [10]), and hence a complete blockage of the pentose phosphate pathway is obviously not taking place.

Citrate is one of the most reputed inhibitors of glycolysis, and the ability of *A. niger* to overproduce citrate by an active glycolytic pathway has attracted biochemical interest for a long time. The explanation in short for this phenomenon is that under appropriate nutrient conditions citrate inhibition is heavily counteracted by the accumulation of various positive effectors, and this feedback does not occur (Habison et al. [11], Arts et al. [12]).

Phosphofructokinase, a key enzyme for glycolysis is regulated by a large number of metabolites, both inhibitory (citrate, phosphoenol-pyruvate [PEP], ATP) and stimulatory (NH_4^+ , AMP, fructose 2,6 bisphosphate [F2,6 P₂]). Allosteric activation of phospho-fructokinase by F2,6 P₂ is probably the most relevant factor in the regulation of the enzyme (Arts et al. [12]). Under physiological conditions phosphofructokinase is not even active in the absence of F2,6 P₂ (Ruijter et al. [13]). In high-producer mutants, phosphofructokinase and hexokinase activities were 2-fold higher than in the parental strain (Schreferl et al. [14]). However, amplification of phosphofructokinase, individually or in combination with pyruvate kinase, did not increase the rate of citric acid production (Ruijter et al. [13]). Interestingly, recombinant strains with increased phosphofructokinase activity consistently had a 40% lower intracellular concentration of F2,6 P₂. *In vitro* measurements mimicking intracellular conditions, assuming an intracellular pH of 6.8, revealed that such a reduction in the F2,6 P₂ concentration could significantly decrease the specific activity of phosphofructokinase in the cell. The level of the enzyme producing F2,6 P₂ (6-phosphofructo-2-kinase) was comparable in parent and recombinant strains, suggesting regulation of its activity. Thus, the fungus seems to adapt to overexpression of phosphofructokinase by decreasing the specific activity of the enzyme through a reduction in the level of F2,6 P₂.

The regulation of phosphofructokinase by F2,6 P₂ may not be the only parameter regulating citrate accumulation. Inhibition of phosphofructokinase by citrate seems *in vivo* also to be antagonized by ammonium ions (Habison et al. [11]). This antagonism is functionally linked to the well-known effect of trace metal ions (e.g. particularly manganese ions) on citric acid accumulation, as one of the effects caused

by manganese deficiency is an impairment of DNA synthesis in *A. niger* (Kubicek et al. [15], Hockertz et al. [16]), which causes increased protein degradation (the effect of manganese deficiency can be mimicked by addition of hydroxyurea, an inhibitor of the enzyme ribonucleotide reductase that provides deoxyribonucleotides for DNA replication). As a consequence, mycelia accumulate elevated concentrations of NH_4^+ . Mutants with partially citrate-insensitive phosphofructokinase exhibited a citrate accumulation more tolerable to the presence of Mn^{2+} (Schrefferl et al. [14]). Also, the precisely controlled exogenous addition of NH_4^+ during particular stages of citrate fermentation even stimulates the rate of citrate production (Yigitoglu & McNeil [17]).

A further mechanism of regulation of phosphofructokinase would involve the phosphorylation of the enzyme by a cyclic-AMP-dependent protein kinase (Legisa & Bencina [18]). They speculate that a high concentration of sucrose induces an increase in mycelial cyclic-AMP levels, which trigger the phosphorylation of phosphofructokinase, thereby converting an inactive (non-phosphorylated) form into an active (phosphorylated) one (Legisa & Gradisnik-Grapulin [19]). The support for their model is their observation that phosphofructokinase was inactivated by treatment with alkaline phosphatase (Legisa & Bencina [18]). Unfortunately, their paper does not indicate whether (and how) the alkaline phosphatase had been removed or inactivated prior to the phosphofructokinase assay. If this has not been done, the inactivation of phosphofructokinase may have been due to a removal of fructose-6-phosphate from the assay and thus be an artefact.

The Cleland-Johnson reaction

Catabolism of glucose via glycolysis yields 2 moles of pyruvate, which are subsequently converted to the precursors of citrate (oxaloacetate and acetyl-CoA). Cleland & Johnson [2] were the first to show that *A. niger* re-uses the CO_2 released in decarboxylation of pyruvate, to form oxaloacetate. This reaction is of utmost importance to the high (80%) citric acid yields commonly obtained, because oxaloacetate could otherwise only be formed by one turn of the Krebs-cycle. This would result in the loss of 2 moles of CO_2 , with a concomitant maximum theoretical yield of 67%. The enzyme catalyzing this reaction is pyruvate carboxylase. Unlike the enzyme from several other eukaryotes, pyruvate carboxylase of *A. niger* is localized in the cytosol (Bercovitz et al. [20], Jaklitsch et al. [21]). Glycolytic pyruvate will therefore be converted to oxaloacetate and reduced to malate by the cytosolic malate dehydrogenase isoenzyme (Ma et al. [22]), thereby also regenerating half of the glycolytically produced NADH. It has been postulated (Kubicek [23]) that in analogy to higher eukaryotes, the cytosolic malate may serve as the co-substrate of the mitochondrial tricarboxylic acid carrier, which probably catalyzes transport of citrate

from the mitochondrion to the cytoplasm. The fixation of CO_2 does not seem to occur during the early phases of fermentation, since in the first 70 hours respiratory coefficient (CO_2 released / O_2 taken up) is close to 1, and it reaches the level predicted from the operation of the pyruvate carboxylase reaction (0.66) only at the producing phase. Hence, the Cleland-Johnson reaction is important in the citrate producing phase only, whereas initial accumulation takes place without anaplerotic CO_2 supply. Interestingly, sparging extra CO_2 into the fermenter to supply pyruvate carboxylase did not result in improved productivity (McIntyre & McNeil [24]).

The role of the Krebs-cycle

Numerous workers claimed that inactivation of citrate-degrading enzymes (aconitase, isocitrate dehydrogenase) should be essential for the accumulation of citric acid. However, solid evidence proves that an intact Krebs-cycle is present throughout the fermentation, hence explanations based on this view are incorrect. The same is true for theories based on the inactivation of these enzymes due to metal ion deficiencies.

Inhibition of the NADP-dependent isocitrate dehydrogenase by glycerol was proposed to decrease flux through the Krebs-cycle and, because of the K_{eq} of aconitase, lead to accumulation of citrate (Legisa et al. [25]). However, studies of increased mycelial glycerol concentrations on the oxidation of 1,5- ^{14}C -citrate indicated that the appearance of labeled CO_2 (which, because of the labeling position applied can only be released during the metabolic conversion of citrate to 2-ketoglutarate) was unaffected by high glycerol concentrations (Arisan-Atac & Kubicek [9]). Moreover, highly purified NADP-specific isocitrate dehydrogenase was found not to be inhibited by citrate (Arisan-Atac et al. [9]).

A reasonable, although so far neglected hypothesis is that a mitochondrial tricarboxylate transporter enzyme is responsible for the accumulation of citrate. This carrier would compete directly with aconitase for citrate and may be able to pump it out of the mitochondria without any necessity for a bottleneck in the Krebs-cycle downstream of citrate. As the tricarboxylate carrier of mammalian tissues and yeast operates by countertransporting with malate (Evans et al. [26]), such a situation is envisagable when its counterion malate accumulates in the cytosol. In fact, malate accumulation has been shown to precede citrate accumulation (Röhr et al. [27]). Nevertheless, the mitochondrial citrate carrier of *A. niger* has not yet been investigated, rendering this intriguing theory a speculation thus far.

The alternative respiratory pathway

Formation of citric acid is dependent on strong aeration, and dissolved oxygen tensions higher than those required for vegetative growth of *A. niger* stimulate production. On the other hand, sudden interruptions in the air supply cause an irreversible impairment of citric acid production without any harmful effect on mycelial growth (Kubicek et al. [28]). An explanation for these observations is that *A. niger* forms a cyanide-resistant, salicylhydroxamic acid sensitive alternative respiratory pathway under conditions that promote citrate production (Fig. 2). In the course of the fermentation the activities of the cytochrome-dependent respiratory enzymes decrease whereas the activity of the alternative oxidase increases. The alternative oxidase does not pump protons concomitantly with the electron transport, and its physiological function is thought to be the removal of the reducing equivalents in excess. The almost quantitative conversion of hexose to citric acid results in net production of 1 mol of ATP and 3 moles of NADH. Since there is not much growth in the stage of citric acid production, the cells do probably not require much ATP, and a switch from cytochrome-dependent respiration to the alternative one would enable the fungus to re-oxidize its NADH without concomitant ATP production. Recently, a cDNA encoding an alternative oxidase was cloned (Kirimura et al. [29]), but it is still not known whether citric acid production rates are affected by the overexpression or disruption of this gene.

The existence of the cyanide-resistant alternative respiratory pathway is known for more than seven decades now, yet its regulation is not fully understood. In plants the intensity of the alternative route will depend on the amount of oxidase present, on the redox status of the redox-sensitive disulphide bond between neighbouring subunits, on the intracellular concentration of organic acids (particularly of pyruvate), and also on the ratio of reduced ubiquinone to the total ubiquinone pool. A conserved cysteine residue near the N-terminus of the protein is responsible for both disulphide bond formation and organic acid activation of the alternative oxidase. Replacing this cysteine with a serine residue results in a permanently monomeric enzyme that is specifically stimulated by succinate at concentrations of 1–5 mM. These concentrations are likely to be physiologically relevant, as succinate is produced within the mitochondrial matrix where it may become concentrated.

It was just recently established that in contrast to plant enzymes, fungal alternative oxidases are permanently monomeric and are not activated directly by α -keto acids. It was also revealed that a domain of about 40 amino acids responsible for the establishment of the disulphide bond in the plant enzyme is missing in these sequences.

In recent years, more and more results suggested that this non-coupled respiration may contribute to the prevention of the generation of reactive oxygen species. These are formed under conditions when the cytochrome path is impaired, and can initiate lipid peroxidation and a subsequent damage in membrane structures. In plants, when substrate supply and hence the reduced ubiquinone-pool is too large in the cytochrome pathway, organic acids, especially pyruvate, accumulate and lower the K_m value of the oxidase for ubiquinol in a classical feed-forward regulation. Should the capacity of the alternative route not be sufficient, active oxygen species themselves induce the expression of the alternative oxidase protein, similarly to the H_2O_2 -mediated expression of the plant pathogenesis-related proteins. In the light of the recently proposed nature of the fungal alternative oxidase, however, reactive oxygen species might be the only signal towards activation (Karaffa et al. [30]). Whether or not the enhanced dissolved oxygen tensions, necessary for high citrate production rate affects the appearance of these molecules and concomitantly that of the alternative oxidase remains to be elucidated.

The alternative oxidase is growth rate dependent and seems functionally connected to the succinic dehydrogenase complex (Karaffa et al. [31, 32]). The latter was indicated by results where the addition of soybean oil had no effect on the total rate of respiration, but it increased the part of respiration which proceeded via the alternative, cyanide-insensitive part in the soybean supplemented cultures.

Export of citric acid from mycelia

Earlier it was suggested that export of citrate through the membrane is due to the large pH gradient between the cytosol and the extracellular medium, and was postulated that citrate efflux from the cell may occur by diffusion of the 2 (-) citrate anion, driven by a gradient (Kontopidis et al. [33]). It was recently shown, however, that citrate export requires ATP and its V_{max} is not strongly affected by the external pH, rendering the diffusion hypothesis rather unlikely (Netik et al. [34]). Citrate export is strongly increased in mycelia grown under manganese deficiency, which is consistent with previous observations, that intracellular concentration of citrate in manganese sufficient and deficient grown mycelia is not strongly different, despite the 5-7 fold higher extracellular levels under the latter conditions (Prömper et al. [35]). The reason for the requirement of manganese deficiency for citrate export may be related to its precisely opposite effect on citrate uptake – only the manganese-chelated citrate can serve as a substrate for the citrate permease (Netik et al. [34]). The reciprocal effect of manganese ions on citrate export and import may also be related to an other effect of manganese deficiency, which is the inhibition of triglyceride and phospholipid synthesis as well as a shift in the ratio of saturated to unsaturated fatty acids of whole

mycelial lipids. The ability of Cu^{2+} ions to antagonize the harmful effect of Mn^{2+} ions may also be related to citrate excretion, since Cu^{2+} ions strongly inhibit the uptake of citric acid. An other possibility is that the effect of Cu^{2+} ions is due to in the competitive inhibition of the Mn^{+} ion uptake in *A. niger*.

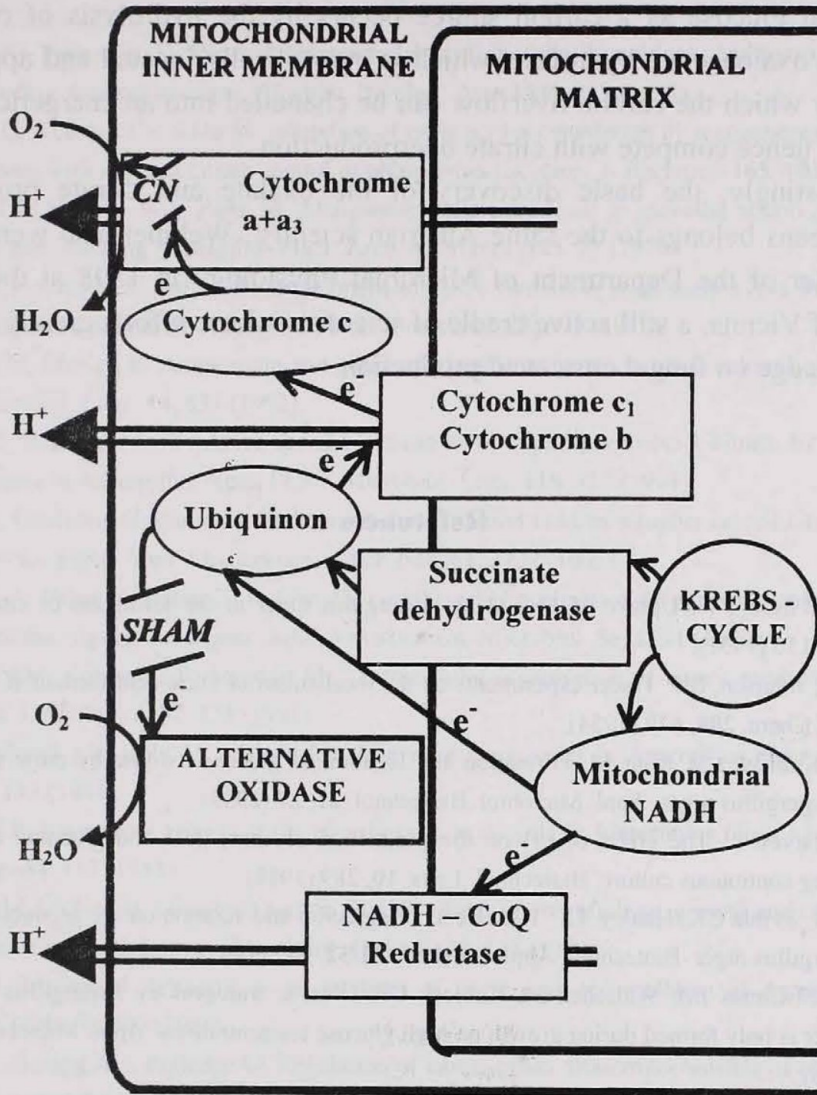


Fig. 2. The alternative respiratory pathway

Oxalic acid biosynthesis

Although not directly within the topic of this lecture, for historical reasons it shall be noted that *A. niger* is also capable of accumulating another organic acid – oxalate – as a (toxic) byproduct of citric acid fermentation. The biosynthesis of this compound on glucose as a carbon source occurs by the hydrolysis of oxaloacetate catalyzed by oxaloacetate hydrolase, which is cytosolically located and appears to act as a valve by which the carbon overflow can be channelled into an energetically neutral pathway and hence compete with citrate overproduction.

Interestingly, the basic discovery of the oxalate and citrate production by microbial means belongs to the same Austrian scientist. Wehmer also went on record as the founder of the Department of Microbial Physiology in 1898 at the Technical University of Vienna, a still active cradle of scientists whose efforts greatly contributed to our knowledge on fungal citric acid production.

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SEXUAL AND ENTERIC BACTERIAL INFECTIONS ELICITING REACTIVE ARTHRITIS

(A REVIEW)*

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In this review synonymous definitions of reactive arthritis are discussed first. Major clinical symptoms, their infectious etiology and epidemiology define post-dysenteric and post-venereal forms of reactive arthritis. Classical (smear, culture, biochemistry, antigen detection) and molecular (DNA and RNA detections) techniques are used in the routine microbial diagnosis that is retrospective in the majority of cases. In the pathomechanism of this disorder, HLA-B27 antigen positivity of patients is a frequent risk factor. Molecular mimicry between microbial and self-antigens, abnormal antigen presentation leading to incomplete CD8+ T lymphocyte activation might contribute to the persistence of microbial antigens that elicit clinical symptoms. Treatment is rarely successful with antimicrobial chemotherapy.

Keywords: reactive arthritis, SARA, HLA-B27, CD8+ T lymphocytes, *Chlamydia trachomatis*, *Yersinia enterocolitica*

Historical background

The occurrence of arthritis following urethral discharge or an acute diarrhea was already mentioned by Hippocrates [1]. Simultaneous occurrence of arthritis and urethritis was described by Alonso Lopez de Hinijos, a Mexican monk in 1578 [2], while arthritis, urethritis and conjunctivitis in the same patient were diagnosed first by Martiniere in 1664 [1] and Stohl in 1776 (AA). Exact scientific description was made

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as “oculo-urethro-synovial triad” or “treponema arthritidis” by Hans Reiter in 1916, what is now called “Reiter’s syndrome” [3]. Specification improved when microbial techniques allowed differentiation of septic from aseptic arthritis. Four cases exhibiting triad symptoms following a *Shigella dysenteriae* outbreak were published by Fiessinger and Leroy [4]. Next, gonococcal infections and in 1950 *Chlamydia trachomatis* infections were recognized as most closely associated with the triad [5]. Post-dysenteric or epidemic Reiter and post-venereal or endemic Reiter syndromes were defined in 1981 [6]. Observing aseptic arthritis after gut infection with *Yersinia enterocolitica*, Aho and coworkers proposed a new definition as “reactive arthritis, (ReA)” that replaced all other terms used previously [7]. In the case of post-venereal forms, “sexually acquired reactive arthritis (SARA)” has become widely accepted [8]. HLA-B27 positivity as a risk factor in patients with ReA after enteral infections was described in 1973 [9].

Definition

Reactive arthritis is defined as aseptic arthritis triggered by an infectious agent located outside the joint. Antigens or nucleic acids of some microbes might be present in the joints. In several patients no triggering agent could be found. All these depend on adequate microbial techniques and now they can be separated from septic arthritides (in which microbes replicate themselves in the synovial fluid). Persisting microbes in the joint (e.g. Lyme disease) also result in the latter form. In rare transient forms seen in HLA-DR4 antigen positive patients, chronic arthritis develops, but neither microbial antigens nor nucleic acids are found by molecular techniques [1].

Major clinical entities leading to reactive arthritis

Neisseria gonorrhoeae infection can be followed by ReA. Non-gonococcal urethritis (NGU) is generally manifested as a mild, non-purulent and painless discharge accompanying or occurring within one month before the onset of arthritis. It may be asymptomatic or accompanied by severe prostatitis. In women, cervicitis is common with vaginal discharge, but sometimes it has no symptoms. *Chlamydia trachomatis* is the most frequently isolated agent [1, 8]. The similar role of mycoplasmas and *Ureaplasma urealyticum* has not been clearly established [1, 10]. SARA develops in 1-3% of NGU patients, of which the majority is male. ReA following acute urogenital symptoms is relatively rare in females. Interestingly enough, sterile urethritis lasting for few days may be a part of post-dysenteric phenomenon [1, 11].

Intestinal symptoms as an acute diarrhea also accompany or within one month precede the onset of arthritis. One to three percents of such patients develop ReA. In general, dysenteric symptoms in these patients are milder than in non-arthritis patients. The incidence of this form of ReA is declining. It is most frequent in age group between 20 to 40 years, and the ratio of male and female subjects is 1:1. The most frequent triggering agents are *Yersinia enterocolitica* (rarely *Y. pseudotuberculosis*), *Salmonella enteritidis*, *typhimurium*, *abony*, *blockley*, *schwarzengrund*, *heidelberg*, *haifa*, *mania*, *newport*, *Clostridium difficile*, *Vibrio parahaemolyticus*, *Shigella flexneri* serotypes 1 and 2 and, *Campylobacter jejuni*, *coli*, *fetus. lari*. They disappear from the bowel when joint symptoms arise, except salmonellae [1].

Screening for microbes

Arthritis develops within 2–3 weeks post infection. Bacterial cultures of synovial fluid and tissues are negative. In the majority of clinical cases, microbial diagnosis is retrospective and based upon tests conducted at time of urogenital or intestinal infections [1, 8, 12]. Steadily increasing number of *Treponema pallidum* infections will bring into focus the syphilis associated ReA again. Seroconversion is proven by Venereal Disease Research Laboratory (VDRL) test, *Treponema Pallidum* Immobilization (TPI) or lately, by Western blot. As *Neisseria gonorrhoeae*, *Salmonella* and *Shigella* species can induce both infectious and reactive arthritides; therefore puncture of synovial cavity is unavoidable to set up exact diagnosis. Smear, cultivation, detection of DNA by polymerase (PCR) or ligase (LCR) chain reactions are the generally used diagnostic methods. In the majority of ReA cases, serovariants D-K of *Chlamydia trachomatis* infect the genitourinary tract, but joints are free of infectious microbes. HLA-B27 antigen positive subjects are more sensitive to chlamydial infection than negative individuals [1, 8, 12]. As exceptions, in few documented cases of ReA, *Chlamydia* could be cultivated from the synovial fluid [8] but their DNA and RNA can be detected regularly by molecular techniques [13, 14]. On the contrary, lipopolysaccharide endotoxin and the outer membrane polypeptide cannot be shown from the synovial fluid. These suggest again that no *Chlamydia* replication takes place in the joints, the microbes are latent. Their antigens may be detected by microimmunofluorescence with specific antisera on urethral or cervical scrapings, while DNA is shown by PCR or LCR. Gene probes are used to detect specific RNA. Antibody quantification has no significant predictive value for ReA. Antibiotic treatment has a beneficial effect if given at the very beginning of the onset of ReA symptoms [15].

Yersinia enterocolitica is the most frequent provocative agent of ReA in Northern Europe, especially in Belgium, where eating raw pork may result in infection. No nucleic acids of it have been detected in the synovial fluid, but polypeptide antigens could be shown [16]. No antibiotic treatment of humans and experimental animals is effective during the course of ReA induced by *Yersinia*, but high dose fluoroquinolones might prevent the onset of ReA symptoms if given in the early phase of gut infection [17–19]. These raise the possibility that *Chlamydia* and *Yersinia* induce ReA in different ways [12]. Stool cultures on selective media, carbohydrate fermentation, biochemical reactions, phage-, bio- and colicin typing, agglutination with specific antisera are the major methods of practical importance to identify *Y. enterocolitica*, *Salmonella* and *Shigella* species, *Vibrio parahaemolyticus* as well. Anaerobic cultivation, detection of A and B exotoxins by neutralization in cell culture, ELISA are used to identify *Clostridium difficile*. *Y. pseudotuberculosis* can be shown in skin biopsy specimen or agglutination is used to quantitate antibodies. Diagnosis of *Campylobacter* species is complemented by dark field microscopy, selective cultivation, oxidase test, and serotyping O and H antigens. Rarely, another microbes also might induce ReA. Hepatitis B is transmitted sexually or by intravenous drug abuse. Screening both antibodies and antigens is carried out by commercial ELISA kits. Herpes simplex virus types 1 and 2 infections are diagnosed by PCR, LCR or cultivation followed by verification through immunofluorescence or molecular methods. So far no ReA has been detected following natural infection with *Shigella sonnei*, *S. typhi*, *E. coli* infections or microbes inducing chronic enteritis [1].

Newly recognized forms of ReA might complicate the picture. Arthritis-dermatitis syndrome occurs in 8 to 36 percent of patients (mostly females) after jejunocolonic bypass surgery in the first three postoperative years [20]. The syndrome is associated with elevated levels of circulating immune complexes to *Escherichia coli*, *Bacillus fragilis* or group D *Streptococcus*.

Whipple's disease is a rare disorder with polyarthritis, which appears to subside several months to 2 years before the onset of diarrhea. Applying PCR, a previously uncultured new bacterium, *Tropheryma whippeli* was discovered as the possible causative agent, that has some homology with the actinomycetes [21].

Pathogenic considerations

The inflammatory nature of ReA is proven by an elevated erythrocyte sedimentation rate, an increased concentration of C-reactive protein. The synovial fluid contains more than 2000 cell/mm³, with a majority of polymorphonuclear leukocytes. Its complement concentration is normal. Periarticular tissues show vascular congestion

and perivascular cell infiltration, mainly with neutrophils. Other histological findings resemble psoriasis [1, 8, 10, 13]. The close but not exclusive association of ReA with HLA-B27 antigen suggests several possible roles as an antigen of MHC class I molecule. In HLA-B27 positive patients, upregulated mechanism of neutrophils might augment inflammatory response [22]. HLA-B27 molecule presents specific peptides to T helper cells, which are recognized as autoantigens by cytotoxic T cells, but antigen presentation seems to be inefficient in ReA patients [12, 23]. HLA-B27 molecule might behave as a receptor by binding bacterial antigens then this complex induces an abnormal immune response [12, 24]. It is also plausible that the amino acid sequence of HLA-B27 peptide and that of certain bacteria have high homology. The human immune system recognizes bacterial components as self – and consequently autoimmune reactions damage the synovium. Partial homology between HLA-B27 antigen and *Yersinia* and *Treponema* species has already been detected [12]. Molecular mimicry between the presented enterobacterial oligopeptide on antigen presenting cells and self-antigens was also shown [12]. Microbes possess thousands of antigens, but those that induce ReA are regarded as dominant antigens. These might be expressed or accumulated in the special microenvironment of the joints. The synovial fluid is acidic and lacks oxygen, consequently favors expression of heat shock proteins (hsp) and cationic polypeptides, which preferably bind HLA-B27 molecules. Some microbial antigens playing a role in the pathogenesis of ReA have already been identified: e.g. hsp57, a 30 kDa protein and a 18 kDa histone-like cationic polypeptide in *C. trachomatis*, and a 61 kDa hsp, a 23 kDa ribosomal and a 19 kDa urease polypeptide in *Y. enterocolitica* [12]. *In vivo* experiments support biochemical data: in HLA-B27 transgenic rats ReA does develop in the presence of normal intestinal flora, but does not in germ-free-animals [26].

Studies on *Yersinia* induced Reiter' syndrome demonstrated low-level initial IgM production with strong IgG and especially IgA response. Several antigenic determinants elicit antibody response with consequent immune complex formation in the serum and synovial fluid. T lymphocytes are activated locally in the joints by antigens: their synovial clonal expansion upon stimulation with antigens of *Chlamydia* and *Yersinia* is stronger than that in the blood. In addition to activated CD45RO⁺ T cells, large amounts of CD45RA⁺ resting cells are found in the synovial fluid. The majority of T cells are CD4⁺ although they are not antigen specific. Oligoclonal CD8⁺ cells present in the synovium might have the major role in the pathomechanism of ReA through cytotoxic activity. *C. trachomatis* is an obligatory, while yersiniae and salmonellae are facultative intracellular parasites. It has been shown that macrophages carrying these bacteria induce a weak CD8⁺ cytotoxic response. The synovial fluid of ReA patients contains high level IL-4 (Th2 type) [27]. All these phenomena might

suggest an incomplete elimination of triggering microbes and their persisting antigens in the joints induce inflammation [10, 12–14, 28].

Microbial differential diagnosis

If gonococcus infection is suspected (usually in women), culture of blood and synovial fluid must be made concurrently. Upon therapeutic intervention the infectious form of arthritis subsides dramatically within few days. HIV infection induces acute but transitory joint pains. Concomitant ReA may be worsened by HIV. Therefore the search for HIV seroconversion is recommended in even typical cases of ReA. In parvovirus arthropaties detection of viral DNA in the serum, lymphocytes, tissues by PCR and IgM/IgG detection by serological tests clarifies origin. Lyme disease is confirmed microbially after detecting IgG/IgM (ELISA, immunofluorescence, Western-blot) or cultivation of *Borrelia burgdorferi*. High dose penicillin treatment promptly relieves symptoms in Lyme disease but is ineffective in real ReA. Beta-hemolytic streptococci cause rheumatic fever. Intravesical injection of bacillus Calmette-Guérin in patients with bladder tumor may induce arthritis. Occasionally brucellae, *Mycobacterium phlei* or parasitic infections are associated with arthritis, but these are cured by a specific treatment, which is not in case of ReA [1, 10, 13].

Antimicrobial treatment

There is no antimicrobial cure for the majority of ReA cases due to retrospective diagnosis. But *C. trachomatis* frequently remains present in patients with urethritis or cervicitis. Combination of trimethoprim and sulfamethoxazole, furthermore tetracycline or ciprofloxacin treatments for 3 months eradicate this microbe with beneficial clinical effects regarding ReA. The number of patients with SARA has significantly decreased in the recent years due to the early treatment of non-gonococcal urethritis. Immunosuppressive therapy may reduce clinical symptoms, but should be avoided in young adults [1, 8].

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MOLECULAR GENETIC AND TRADITIONAL METHODS FOR DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX (DISCREPANCY ANALYSIS)*

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In the past six and half years, 862 different clinical samples [sputum, bronchoalveolar lavage, thorax puncture, cerebrospinal fluid and skin samples] were tested by Gen-probe amplified *Mycobacterium tuberculosis* direct test (MTD) or ligase chain reaction (LCR) or polymerase chain reaction (PCR). 239 parallel clinical samples were cultivated, and some samples were stained with Ziehl-Neelsen staining. 1–4 samples were tested per patient. 29 (12.13%) samples were positive and 177 (74.05%) samples were negative with both cultivation and molecular genetic methods. 2 (0.83%) samples were positive only on cultivation, and 31 (12.97%) samples were positive only with the molecular diagnostic methods. The differences are undoubtedly explained by the sensitivity of the molecular diagnostic methods.

Keywords: tuberculosis, MTD, LCR, PCR, discrepancy analysis

Introduction

Microbiological diagnostics has been reformed during the past 15 years by the introduction of molecular biological methods. Microbial pathogens, bacteria, viruses and fungi can now be detected within some hours with molecular diagnostic techniques. This was not possible with traditional methods, or only after a long culturing time, as in case of *Mycobacterium tuberculosis* (*M. tuberculosis*).

* This paper was written to commemorate to the fiftieth anniversary of the foundation of the Hungarian Society for Microbiology.

The methods for the diagnosis of the *M. tuberculosis* complex have not progressed for decades because of the long cultivation time. The increases in AIDS opportunistic mycobacterial infections and the number of multiresistant strains have made it urgent to elaborate rapid diagnostic methods to detect the *M. tuberculosis* complex [1]. The development of amplification methods was a very important step forward in this field.

New radiometric liquid cultivation methods [BACTEC], and new rapid diagnostic methods have been introduced in recent years, e.g. chromatographic methods [HPLC and GLC], nucleic acid hybridization and amplification methods for diagnosis of the *M. tuberculosis* complex [2–5].

There are three different traditional levels of *Mycobacterium* diagnostics in Hungary: direct detection of the bacterium by acid-fast Ziehl-Neelsen staining, cultivation of the bacterium in regional centers, and identification and antibiotic-sensitivity testing methods in the national center.

The conventional cultivation method, which is regarded as the "gold standard" [Löwenstein-Jensen] and Gen-probe amplified *Mycobacterium tuberculosis* direct test (MTD) or ligase chain reaction (LCR) or polymerase chain reaction (PCR) were compared in our laboratories [6]. The members of the *M. tuberculosis* complex are *M. tuberculosis*, *M. bovis* BCG, *M. bovis*, *M. africanum* and *M. microti*, all of which are detected by MTD, while all except *M. bovis* BCG are detected by LCR and PCR.

Materials and methods

The clinical samples were digested and decontaminated by the NALC-NaOH method. The decontaminated samples were cultivated at 37°C for 6 or more weeks, and examined weekly.

The MTD method utilizes a proprietary isothermal enzymatic amplification of target rRNA via DNA intermediates. The detection of amplified product is achieved by using an acridine ester-labeled DNA probe [7]. The major processes of *M. tuberculosis* MTD are as follows: sonication breaks the mycobacterial cell wall, liberating rRNA, which is added to the amplification mix. This mix contains the nucleotides, the primers and enzymes. In the second step, the primers anneal to target RNA sequences. The reverse transcriptase and nucleoside triphosphates initiate the DNA synthesis. Subsequently, the reverse transcriptase converts cDNA:RNA duplex into template for RNA synthesis, and the transcriptase makes many RNA copies. The chemiluminescent-labeled hybridized DNA/RNA complexes were detected in a Leader 50 luminometer.

The LCR is an amplification method [8]. The four oligonucleotide probes recognize and hybridize to a specific target sequence within the chromosomal DNA of

the *M. tuberculosis* complex. The oligonucleotides are designed to be complementary to the target sequence so that in the presence of target, the probes will bind adjacent to one another. They can then be enzymatically joined to form the amplification product, which subsequently serves as an additional target sequence during further rounds of amplification. After sufficient numbers of target amplification product have accumulated, they can be detected by microparticle enzyme immunoassay (MEIA) that is measured by the MEIA optical assembly.

The Amplicor PCR assay includes three major steps: PCR target amplification, hybridization of the amplified product to a specific nucleic acid probe and colorimetric detection. Genus-specific primers located in a highly conserved region of 16S rRNA *Mycobacterium* gene are used to amplify a 584-bp sequence. Carry-over contamination in the Amplicor PCR test is prevented by incorporating dUTP [9-11]. The automatized Cobas Amplicor system was used for examination of clinical samples.

Results

862 different clinical samples [sputum, bronchoalveolar lavage (BAL), thorax puncture, cerebrospinal fluid (CSF) and skin] were tested by the MTD, LCR or PCR and cultivation methods, and some samples were subjected to Ziehl-Neelsen staining. 39 (4.52%) sputum samples, 27 (3.13%) BAL samples, 9 (1.04%) thorax puncture samples, and 12 (1.39%) other samples were positive among the 862 samples (Table I).

Of the 459 clinical samples from males 66 (7.65%) were positive. Of the 403 clinical samples from females 21 (2.44%) were positive (Table II).

1-4 samples were tested per patient. 239 (27.73%) of 862 amplified and cultivated samples were compared. 29 samples (12.13%) were positive and 177 (74.05%) samples were negative by both the cultivation and the amplified methods. 2 (0.83%) samples were positive only on cultivation, and 31 samples (12.97%) were positive only with the amplified methods. The parallels of amplified positive samples were negative on cultivation in a higher percentage, whereas the clinical symptoms and results of the amplified methods generally correlated. Comparative results on 26 patients are given in Table III.

The different cultivation and amplification results are explained in Table IV. The differences in the first four cases are undoubtedly explained by the sensitivity of the amplification methods. For patients 5, 24 and 25 different samples were processed for the culture and molecular genetic methods. For patient 6, carcinoma was diagnosed on the basis of the clinical, X-ray and CT examinations.

Table I

Distribution of results on clinical samples for detection of M. tuberculosis complex between 01 July 1994 and 31 December 2000

	Sputum N (%)	BAL N (%)	Thorax puncture N (%)	Others N (%)	Total N (%)
Positive	39 (4.52)	27 (3.13)	9 (1.04)	12 (1.39)	87 (10.09)
Negative	201 (23.32)	156 (18.09)	51 (5.92)	367 (42.58)	775 (89.91)
Total	240 (27.84)	183 (21.23)	60 (6.96)	379 (43.97)	862 (100.0)

Table II

Distribution of clinical samples by sex between 01 July 1994 and 31 December 2000

	1994 N (%)	1995 N (%)	1996 N (%)	1997 N (%)	1998 N (%)	1999 N (%)	2000 N (%)	Total N (%)
Male positive	20 (2.32)	17 (1.97)	13 (1.51)	6 (0.69)	4 (0.46)	0 (0.00)	6 (0.69)	66 (7.65)
Female positive	1 (0.12)	6 (0.69)	2 (0.23)	2 (0.23)	4 (0.46)	3 (0.35)	3 (0.35)	21 (2.44)
Male negative	16 (1.86)	70 (8.12)	77 (8.93)	63 (7.31)	48 (5.57)	48 (5.57)	71 (8.24)	393 (45.59)
Female negative	5 (0.58)	27 (3.13)	64 (7.43)	61 (7.08)	73 (8.47)	67 (7.77)	85 (9.86)	382 (44.32)
Total	42 (4.87)	120 (13.92)	156 (18.10)	132 (15.31)	129 (14.97)	118 (13.69)	165 (19.14)	862 (100.0)

After lobectomy, the morphological results confirmed this diagnosis. For patient 7, on the basis of the X-ray and molecular genetic results antituberculous treatment was started in spite of a negative culture result. This homeless and alcoholic patient was released after 6 months of anti-TB treatment, with X-ray regression. For patients 8 and 9, the culture became positive after 10 weeks (Table IV).

Table III

Discrepancies in detection of Mycobacterium tuberculosis complex (N 25)

Patients	Samples (N)	Cultivation positive (N)	Molecular genetic positive (N)
1	4	1	3
2	4	1	4
3	2	1	2
4	3	1	3
5	1	0	1
6	2	0	1
7	1	0	1
8	2	0	2
9	1	0	1
10	2	1	2
11	2	0	2
12	2	0	1
13	1	0	1
14	3	0	3
15	1	0	1
16	1	0	1
17	1	0	1
18	1	0	1
19	1	0	1
20	1	0	1
21	1	0	1
22	1	0	1
23	1	0	1
24	1	1	0
25	1	1	0

Discussion

The conventional cultivation methods are time-consuming. The cultivation, identification and antituberculous drug sensitivity testing requires weeks or months in some cases.

The amplification methods require only some hours after decontamination. Detection of the *M. tuberculosis* complex by MTD involves the detection of living bacteria in clinical samples. The procedure is suitable for detection of the *M. tuberculosis* complex in immune complexes and white blood cells too.

The cultivation method is not indispensable because of drug sensitivity testing. The costs of the amplification methods are apparently high, but the benefit is really much higher, considering the advantages of the rapid diagnosis: the beginning of immediate therapy, the prevention of further mycobacterial infections and the lower hospital hotel costs [12-14]. In consequence of their sensitivity, molecular diagnostic methods are able to amplify the nucleic acid of 1-2 bacteria, even if the patient has been treated with antibiotics in the meantime [15].

Table IV

Explanation of the different culture and molecular genetic results

Cases	Explanation
1-4, 10-23	The differences are undoubtedly explained by the sensitivity of the molecular diagnostic methods.
5, 24-25	Different samples were processed for cultivation and molecular genetic methods
6	Carcinoma was diagnosed on the basis of clinical, X-ray and CT examination. After lobectomy, the morphological results confirmed the diagnosis.
7	On the basis of clinical, X-ray and molecular genetic results, antituberculous treatment was started in spite of a negative culture result. This homeless and alcoholic patient was released after 6 months of anti-TB treatment, with X-ray regression.
8, 9	The culture became positive after 10 weeks.

The development of amplification methods was a very important step forward in this field. The application of rapid diagnostic methods in mycobacteriology was emphasized by several unsolved problems. The introduction of amplification methods (MTD, PCR, LCR, and NASBA) has opened up new dimensions in mycobacterial diagnostic methods.

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PHENOTYPIC AND GENOTYPIC PROPERTIES OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* STRAINS ISOLATED IN HUNGARY, 1997–2000*

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An account is given on the activity of the National Center for Phage Typing of Staphylococci in Hungary in the period between 1997 and 2000 related to methicillin resistant *Staphylococcus aureus* (MRSA) strains originating mainly from hospital infections and sporadic cases. The rate of multiresistant MRSA strains has decreased gradually from 98.1% in 1997 to 74.6% in 2000, accordingly the typability by phages showed a considerable improvement by the international basic phages. Resistance pattern of MRSA strains became narrower in the period of the examinations. With the exception of erythromycin the rate of resistance decreased probably as a consequence of the increased use of erythromycin. The typing method was completed with the phenotypic and genotypic characterization of macrolide resistance. Among 73 MRSA strains type A was the most frequent macrolide resistance group, while type B, C1 and C2 occurred rarely. Type A was frequent also among the few MSSA and CNS strains. Out of the 168 examined *S. aureus* strains *ermA* genes occurred in 81.5%; in MSSA and CNS strains *ermC1* genes were frequent, both genes are responsible for the target modification. The *msrA* gene, encoding the increased efflux, occurred only in CNS strains. Comparing the results obtained by phenotyping (phage typing) and genotyping (AP-PCR) methods it is of note that MRSA strains which proved non-typable by phage typing gave suitable results by the AP-PCR.

Keywords: MRSA strains, phage typing, genotyping, antibiotic resistance

* This paper was written to commemorate to the fiftieth anniversary of the foundation of the Hungarian Society for Microbiology.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains were first described in 1961 as "Celbenin" resistant Staphylococci [1]. Their clinical and epidemiological significance has increased gradually since 1980 in the European countries [2–5], in the USA [6] and in Australia [7]. In the 1990s MRSA has become worldwide the most significant nosocomial pathogen [8–12]. Examinations on the virulence of MRSA and methicillin sensitive *S. aureus* (MSSA) led to various conclusions. While in Hong Kong it was found that MRSA and MSSA strains had similar virulence [13], comparison of mortality rate in nosocomial bacteraemia with MRSA and with MSSA showed that it was three times higher in patients of MRSA group [14]. It can be stated that MRSA infections usually cause serious infections, leading to difficulties in their treatment because the strains are not only resistant to all the β -lactam antibiotics, their resistance rates to other antibiotics are also high [15, 16], and in the recent years low-level resistance has emerged even to glycopeptides, vancomycin and teicoplanin [17, 18]. With regard to the serious infections and dangerous nosocomial outbreaks it is important from epidemiological point of view to determine the type of the MRSA strains with a suitable typing method.

In this paper an account is given of investigations on the phenotypic and genotypic properties of MRSA strains isolated from nosocomial infections and from sporadic cases in Hungary between 1997 and 2000.

Materials and methods

Staphylococcal strains

In the period between 1997 and 2000 altogether 3407 *S. aureus* (2693 MSSA, 698 MRSA and 16 coagulase negative Staphylococci /CNS/) were isolated from different nosocomial or sporadic infections in the County Institutes of National Public Health and Medical Officer Services (NPHMOS). For the macrolide resistance studies the following strains were selected: 145 MRSA, 18 MSSA and 16 CNS.

Phenotyping

Staphylococcal phages used for typing

International basic set for phage typing *S. aureus*: 29, 52, 52A, 79, 80(I), 3A, 3C, 55, 71 (II), 6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85(III); 94, 96(V), 81, 95 (Misc) (originating from Public Health Laboratory Service (PHLS), Colindale, London [19,

20]) and the MRSA type phages: 616, 617, 618, 620, 622, 623, 625, 626, 629, 630 [21] were used. Phage typing was carried out by the method of Blair and Williams [22].

Antibiotic susceptibility testing methods

Oxacillin sensitivity was determined by disc diffusion [23] using Mueller-Hinton medium (Oxoid). The disc contained 10µg oxacillin (Human, Budapest). In addition to oxacillin the following antibiotics were tested: ampicillin (AM), streptomycin (SM), tetracycline (TE), gentamicin (GM), vancomycin (VA), erythromycin (EM), oleandomycin (OL), lincomycin (LI), spiramycin (SP).

The resistance phenotype of macrolide-lincosamide-streptogramin B resistance (MLSR) Macrolide type

The placing of the EM, OL, SP and LI antibiotic discs were carried out according to Jánosí's method [24]. Evaluation of and conclusion to the constitutive or inductive character of the resistance was made according to Weisblum [25–26]. On the basis of these characteristics there are 5 distinguishable phenotype classes: A, B, C1, C2 and other.

The macrolide phenotypes group A: constitutive MLSR strains; group B: inducible (partial) PMSR strains; group C1: EM inducible MLSR strains; group C2: EM, OM inducible MLSR strains [27–29].

Genotyping

Arbitrarily primed-PCR (AP-PCR)

AP-PCR typing was carried out according to Welsh and McClelland [30] with slight modifications. DNA preparation and purification was made by Promega Wizard genomic DNA purification kit, according to the manufacturer's protocol.

The amplification was made in Techne Progene PCR apparatus during 42 cycles through the following temperature profiles: 96°C 5 min, after two cycles: 96°C 1 min, 40°C 1 min and 72°C 1 min continued in 40 "standard" cycles: 96°C 1 min, 60°C 1 min and 72°C 1 min. The amplified DNAs were separated by agarose gel electrophoresis and ethidium bromide staining was made during 15 min. The photography was made under UV transilluminator and the evaluation by Gel-Doc 2000 documentation apparatus (Bio-Rad) by the aid of Quantity One evaluation program.

As subtype of the same type of an isolate was regarded when there was one band difference among the patterns [31].

Detection and identification of macrolide resistance genes in MRSA, MSSA and CNS strains

Demonstration of erm genes encoding target modification

Oligonucleotide primer pairs: *ermA*-F 21 nucleotides, *ermA*-R 24 nucleotides [32], *ermB*-F 21 nucleotides, *ermB*-R 24 nucleotides [33], *ermC*-F 21 nucleotides, *ermC*-R 24 nucleotides [34].

Demonstration of genes encoding increased efflux (from S. epidermidis strains)

Oligonucleotide primer pairs: *msrSA*-343F 20 nucleotides, *msrSA*-1237 – 21 nucleotides [35].

Primers were selected as described by Sutcliffe [36] and Nakajima [37]. Specificity of different primers were tested using DNA extracts of the reference strains containing: *ermA* gene (chromosome): *S. aureus* ISP 447; *ermB* gene (plasmid): *S. aureus* 8325 (pI258); *ermC* gene (plasmid): *S. aureus* MS 13837; *msrSA* gene (plasmid): *S. aureus* 8325 (pMC 38).

Preparation of template DNA

A single colony of each isolate was picked up from an agar plate and resuspended in 100 µl of distilled H₂O, and boiled for 10 min. They were placed to -70°C from 2 to 24 hours. The supernatant was collected after spinning for 2 min. in a microcentrifuge.

The DNA concentration of boiled extracts was determined at 260 nm (Genesis UV Spectrophotometer) [38]. Phenol-chloroform extracted DNAs were prepared as described by Kado and Liu [39] and Katsurashima [40]. Purification of DNA was made by Wizard Kit (Promega), as described in the "Protocol".

Amplification protocol

PCR was carried out in 50 µl volumes that contained 20 µg bacterial genomic DNA or boiled extracts for each resistant determinant.

The "master" contained: PCR Buffer (Promega), 200 µM dNTP, 1.5 mM MgCl₂, 0.5 µM primer, 1.25 U Taq.

A Progene (Techne) thermal cycler was used for 1 cycle 94°C 2 min 15 s and 30 cycles of amplification (94°C, 45 s, 55°C, 30 s, 72°C, 1 min 45 s and then 1 cycle 72°C, 10 min). The amplified DNA was separated in 15 µl aliquots and electrophoresed in 1.6% agarose gels containing 0.5 µg/ml ethidium bromide and photographed under UV light.

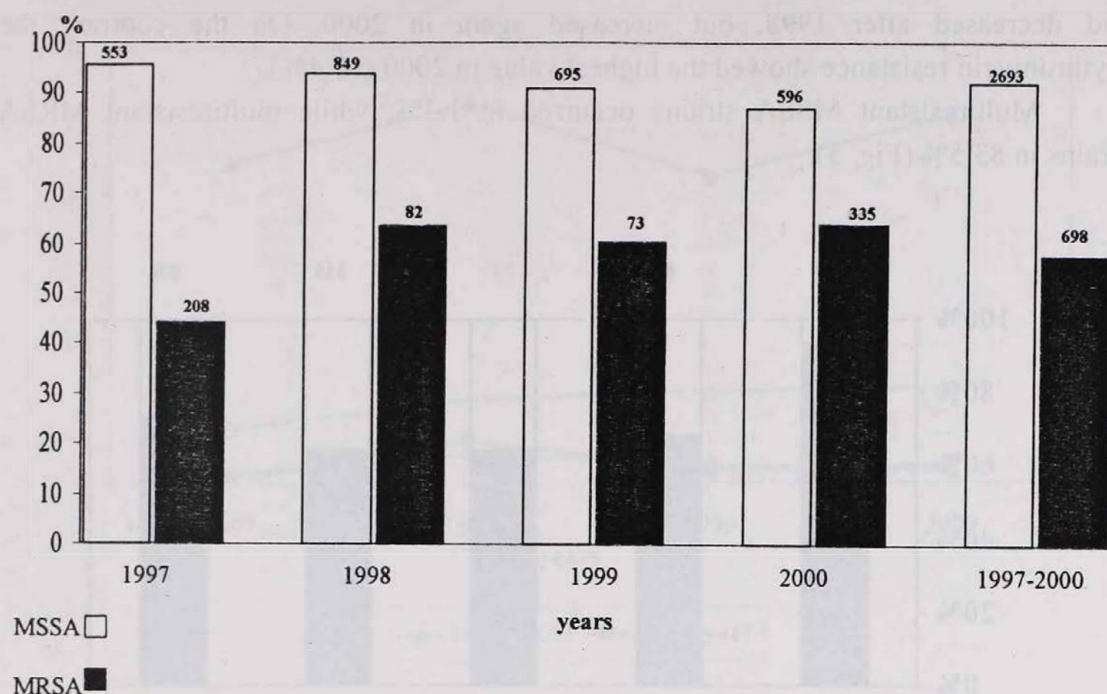


Fig. 1. Typability of MSSA strains by international basic phages

Results

Phage pattern by basic and MRSA phages

Typability of 3391 *S. aureus* strains (2693 MSSA and 698 MRSA) by basic and MRSA phages is shown in Fig. 1 and Fig. 2. MSSA strains were typable by basic phages in 91.8%, MRSA strains in 57.6%, while by MRSA phages in 73.5%.

Antibiotic resistance of MSSA and MRSA strains

Antibiotic resistance of the MSSA and MRSA strains examined in 1997–2000 are demonstrated in Fig. 3 and Fig. 4. Among the 3391 *S. aureus* strains 20.6% were oxacillin resistant. Oxacillin resistant MSSA strains occurred annually between 8.8% and 36.0%. Tetracycline resistance of MSSA strains varied between 21.0% and 26.1%, gentamicin resistance between 0.5% and 1.9%; streptomycin resistance between 0.5% and 2.9%, erythromycin resistance between 3.8% and 6.5%. Antibiotic resistance of MRSA strains showed significantly higher values: tetracycline, gentamicin and streptomycin resistance was the highest in 1997 (92.3%, 90.3%, 90.3%, respectively)

and decreased after 1998, but increased again in 2000. On the contrary the erythromycin resistance showed the highest value in 2000 (93.4%).

Multiresistant MSSA strains occurred in 1.3%, while multiresistant MRSA strains in 83.5% (Fig. 5).

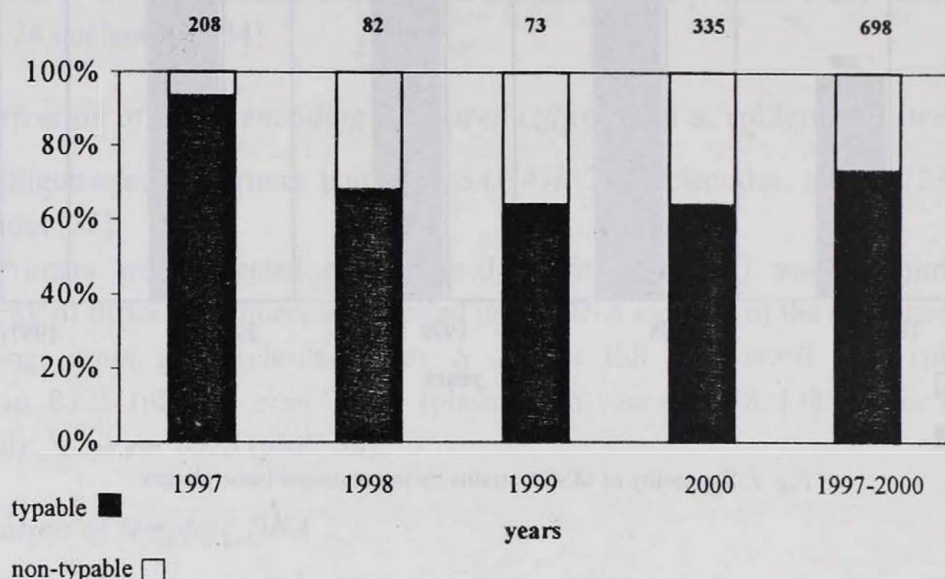


Fig. 2. Typability of MRSA strains by international MRSA phages

Antibiotic resistance according to phage type pattern

Occurrence of antibiotic resistance proved to be different according to phage type pattern and the year of examinations. In case of 83A complex tetracycline resistance e.g. occurred in 1997, 1998, 1999 and 2000 in 28.5%, 10.0%, 55.9% and 49.6%, respectively. Non-typable strains showed the highest antibiotic resistance in every year with all the examined antibiotics (Fig. 6).

Macrolide resistance types

For identification of macrolide type 107 erythromycin resistant MRSA, MSSA and CNS strains were selected. Among 73 MRSA strains type A was found in 91.8%, type B in 2.7%, C1 in 4.1% and C2 in 1.4%. Out of 18 MSSA erythromycin resistant strains 9 strains were of type A, 8 strains were of macrolide type C2 and one of type C1. Out of the 16 CNS strains 11 were of macrolide type A, two strains of type C2 and three strains were non-typable (Table I).

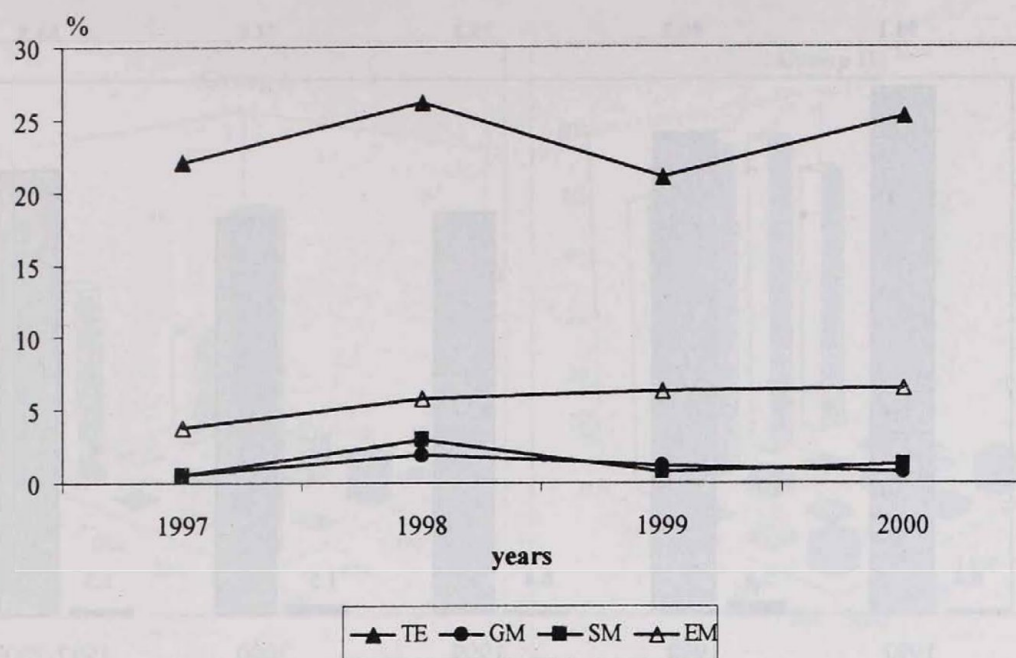


Fig. 3. Antibiotic resistance of MSSA strains

TE=tetracycline, GM=gentamicin, SM=streptomycin, EM=erythromycin

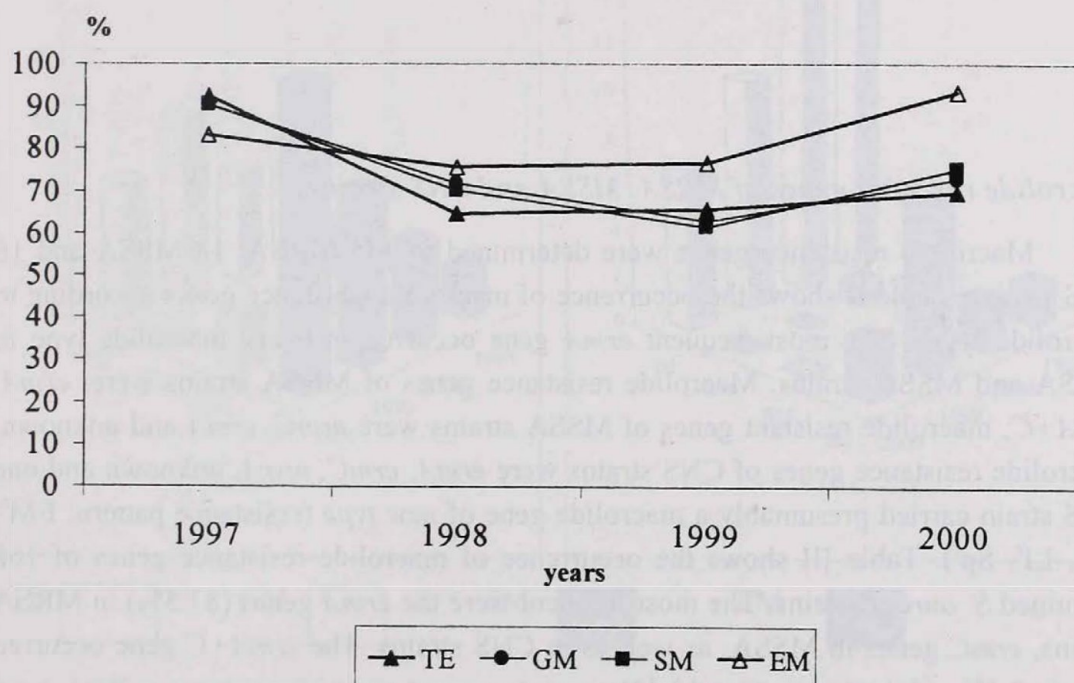


Fig. 4. Antibiotic resistance of MRSA strains. For abbreviations see Fig. 3

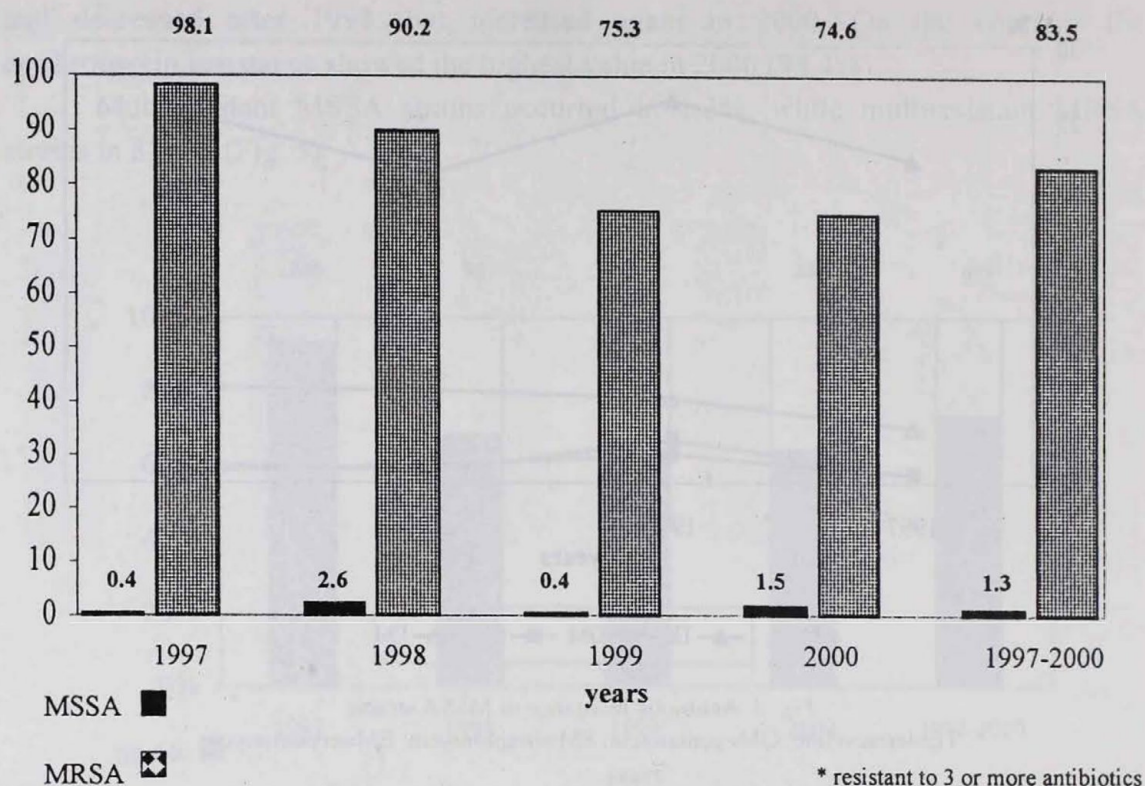


Fig. 5. Multiresistance of MRSA and MSSA strains

Macrolide resistant genes of MRSA, MSSA and CNS strains

Macrolide resistance genes were determined in 145 MRSA, 18 MSSA and 16 CNS strains. Table II shows the occurrence of macrolide resistance genes according to macrolide types. The most frequent *ermA* gene occurred in every macrolide type in MRSA and MSSA strains. Macrolide resistance genes of MRSA strains were: *ermA*, *ermA*+*C*; macrolide resistant genes of MSSA strains were *ermC*, *ermA* and unknown. Macrolide resistance genes of CNS strains were *ermA*, *ermC*, *msrA*, unknown and one CNS strain carried presumably a macrolide gene of *new type* (resistance pattern: EM^s, OL^r, LI^r, Sp^r). Table III shows the occurrence of macrolide resistance genes of 168 examined *S. aureus* strains. The most frequent were the *ermA* genes (81.5%) in MRSA strains, *ermC* genes in MSSA, as well as in CNS strains. The *ermA*+*C* gene occurred only in 2.4%, while the *ermC* in 13.1%.

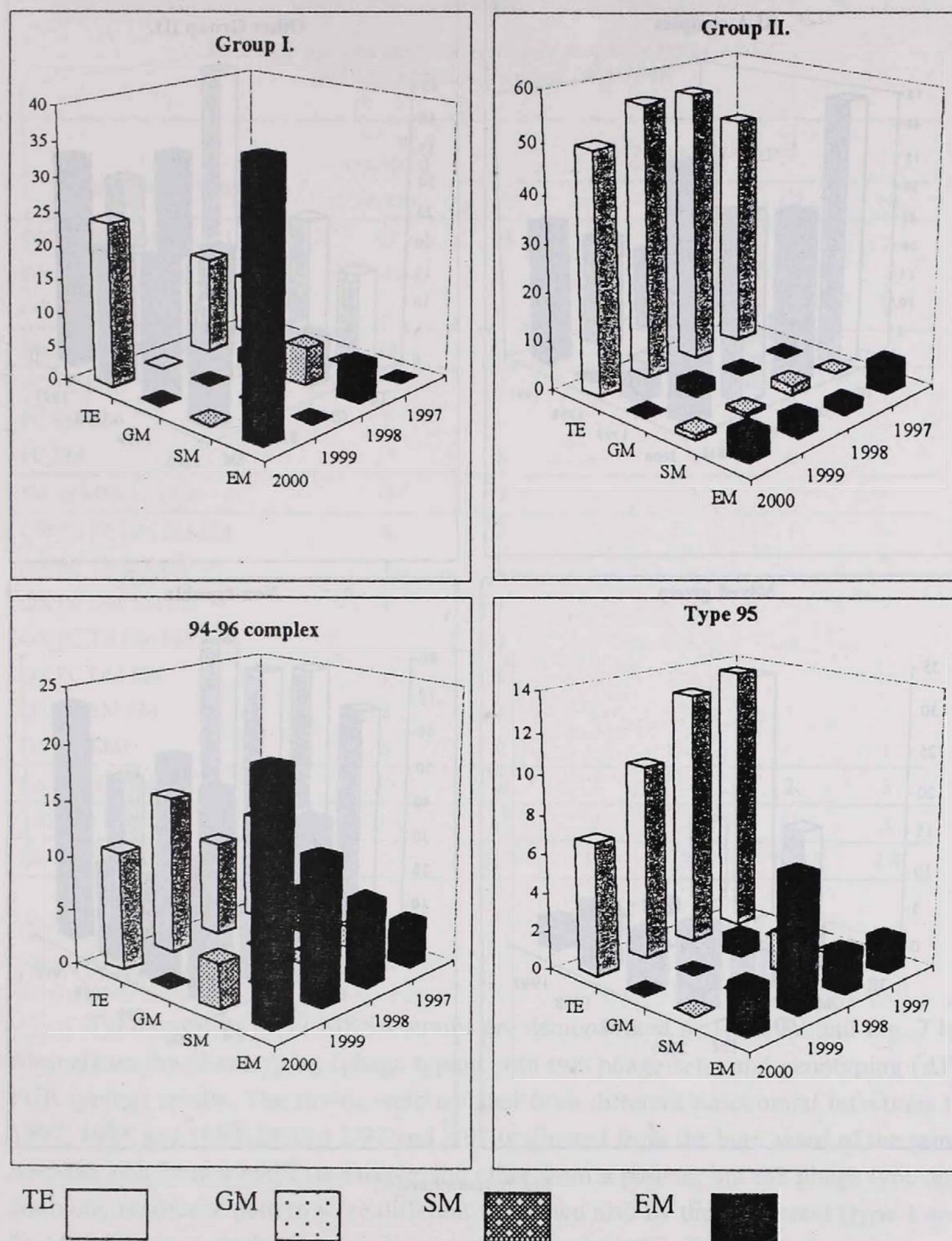


Fig. 6. Antibiotic resistance of frequent *S. aureus* phage groups or type patterns. For abbreviation see Fig. 3

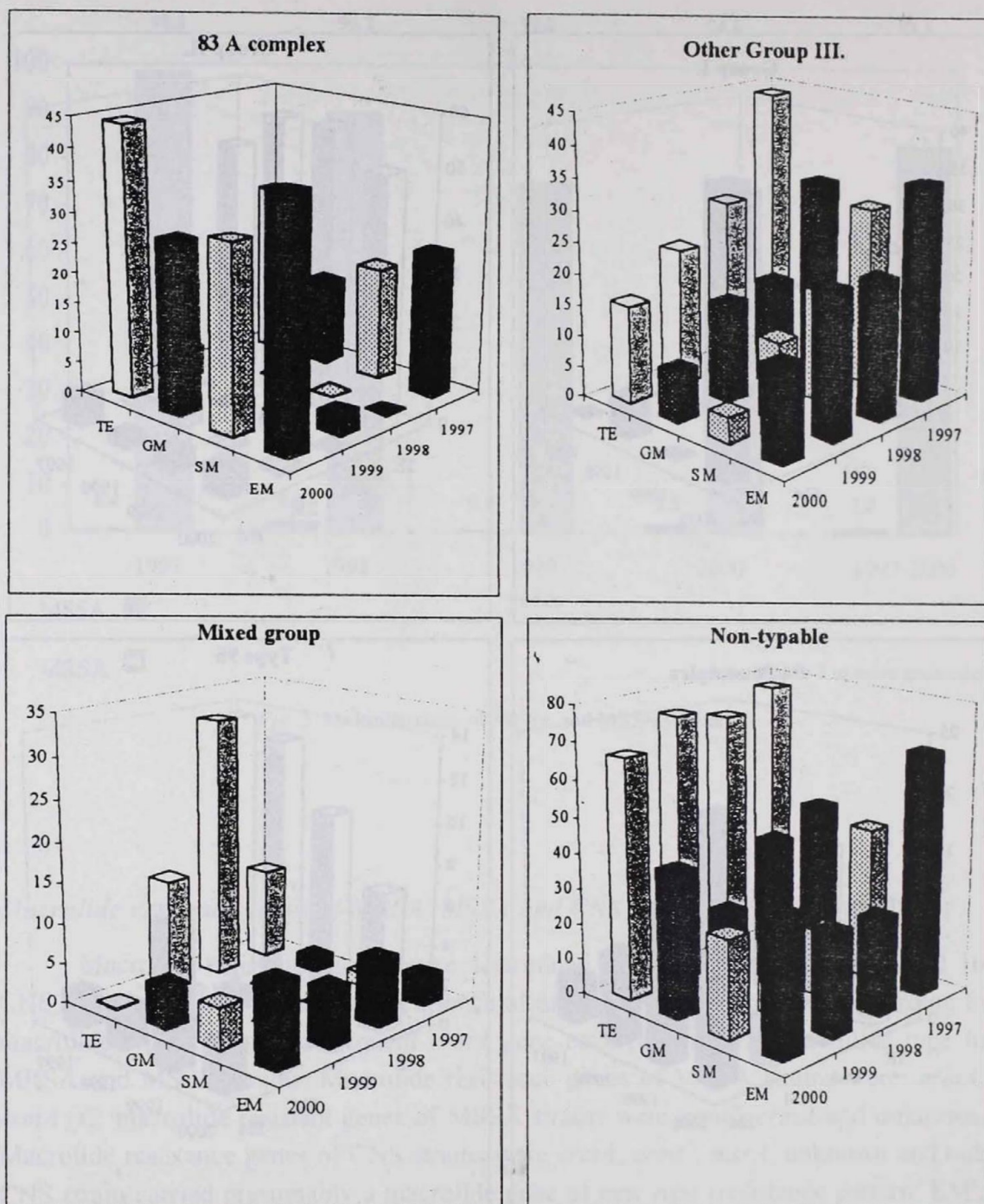


Fig. 6. (continued)

Table I

Macrolide type and antibiotic resistance pattern of MRSA, MSSA and CNS strains in Hungary, 1997–1999

Antibiotic resistance	No. of examined Strains	No. of macrolide types				
		A	B	C1	C2	Nt
OX PC TE GM SM EM	67	65	–	1	1	–
OX PC TE GM EM	4	1	2	1	–	–
OX PC EM	2	1	–	1	–	–
No. of MRSA strains	73	67	2	3	1	–
PC TC EM	2	1	–	–	1	–
PC SM EM	1	–	–	–	1	–
PC EM	15	8	–	1	6	–
No. of MSSA strains	18	9	–	1	8	–
OX PC TE GM SM EM	3	2	–	–	1	–
OX PC TE SM EM	4	2	–	–	–	2
OX PC GM SM EM	1	1	–	–	–	–
OX PC TE GM EM	1	1	–	–	–	–
OX PC GM EM	1	1	–	–	–	–
OX PC SM EM	3	2	–	–	1	–
OX PC EM	3	2	–	–	–	1
No. of CNS strains	16	11	–	–	2	3
Total No of strains	107	87	2	4	11	3
Macrolide types in %		81.3	1.9	3.7	10.3	2.8

AP-PCR types of MRSA strains

Typing results of 12 MRSA strains are demonstrated in Table IV and Fig. 7 by comparison the phenotyping (phage typing with two phage-sets) and genotyping (AP-PCR typing) results. The strains were isolated from different nosocomial infections in 1997, 1998 and 1999. Strains 1/97 and 2/97 originated from the burn ward of the same hospital, one from a hygienic sample, the other from a patient, but the phage type and antibiotic resistance pattern were different supported also by their different (type 1 and 2) AP-PCR types. In the traumatology ward of hospital "E", three MRSA strains were isolated from three different patients (6/97, 7/97, 8/97).

Table II

Macrolide resistance genes of MRSA and CNS strains according to macrolide types

		MRSA (145)								MSSA (18)				
Macrolide type (MT)		A		B2	C1	C2	ND			A	C1	C2		
No. of MT		67		2	3	1	72			9	1	8		
Macrolide resistance gene (MRG)		ermA	erm A+C	ND	erm A	Erm A	erm A	erm A	erm A+C	erm C	ND	erm A	erm C	UK
No. of MRG		59	2	6	2	3	1	70	2	8	1	1	7	1

		CNS (16)							
Macrolide type (MT)		A		C2		NT			
No. of MT		11		2		3			
Macrolide resistance gene (MRG)		erm A	erm C	ND	msr A	UK	msr A	new type	ND
No. of MRG		1	7	3	1	1	1	1	1

Two strains isolated from two different patients were identical with each other according to phage type, antibiotic resistance pattern and AP-PCR type (type 1) alike. The phage type (by MRSA phages) of the third strain originating from the third patient (8/97) proved to be not definitely different, but the AP-PCR typing gave different result (type 2). In the surgery of Hospital "F" one strain was isolated from wound infection of a patient (9/98), and three strains from wound, throat and nose swab samples of another patient (10/98, 11/98, 12/98). These latter strains were not typable by basic phages, but proved to be identical by MRSA phages and were identical or nearly identical by AP-PCR typing (type 1 and 1/1). A strain from the wound infection of the other patient (9/98) proved to be different from the former strains by AP-PCR typing (type 5). Strains 3/99, 4/99 and 5/99 originated from sporadic cases in different hospitals and proved to be different from each other based on phage typing and AP-PCR typing alike (types 1, 3 and 4).

Table III

Multiresistance of MSSA and MRSA strains*

Year	Staphylococci	No. Of strains	Multiresistant strains	
			No	%
1997	MSSA	553	2	0.4
	MRSA	208	204	98.1
1998	MSSA	849	22	2.6
	MRSA	82	74	90.2
1999	MSSA	695	3	0.4
	MRSA	73	55	75.3
2000	MSSA	596	9	1.5
	MRSA	335	250	74.6
1997	MSSA	2693	36	7.3
-2000	MRSA	698	583	83.5

*resistant to 3 or more antibiotics

Discussion

For many years phage typing was the method of choice for investigation of MRSA epidemiology since its discriminatory capability had been demonstrably higher than that of other phenotypic tests [41]. Since 1974 there have been valuable data on the occurrence of MRSA strains in Hungary [42], and since 1990 on their multiresistance and typability by phages [43]. The epidemiological usefulness of phage typing was indisputable also in Hungary [44]. Nevertheless the method requires a large number of typing phages, so it is labour consuming and difficult to standardize. Antibigrams are inadequate for differentiation purposes. Among genotypic methods the plasmid analysis was the first method applied widespread. Many reports indicated the usefulness of plasmid analysis, but the method had its limitations, e.g. similar, but not identical patterns and isolates without plasmid could not reliably be distinguished from similar isolates. Complex typing (phage typing and plasmid profile) of MRSA was applied by Kozarsky et al. [45] and Witte et al. [46].

Following the first molecular technique (plasmid analysis), various molecular methods have been developed and used for typing of MRSA strains. Comparative investigations of traditional and molecular methods for typing isolates of *S. aureus*,

especially of phage typing and pulsed-field gel electrophoresis (PFGE) showed the superiority of PFGE typing [47].

Table IV

Phage types and AP-PCR type of MRSA strains

Strain No./ year	Origin	Test sample	Phage pattern by		AP-PCR type	Antibiotic resistance pattern
			Basic phage-set	MRSA phage set		
1 /97	Hospital "A" Burn ward	nose	NT	630	1	OX PC SM TE GM EM OL LI SP
2 /97	Hospital "A" Burn ward	hygienicsample	75 (III)	618/623/630	2	OX PC TE GM EM OL LI SP
3 /99	Hospital "B" Accident surgery	wound	NT	NT	1	OX PC SM TE GM EM OL LI SP
4 /99	Hospital "C" Vascular surgery	wound	3C (II)	NT	3	OX PC TE
5 /99	Hospital "D" Otolaryngology	throat	116 (II)	NT	4	OX PC EM OL LI SP
6 /97	Hospital "E" Traumatology	nose	NT	622/630	1	OX PC SM TE GM EM OL LI SP
7 /97	Hospital "E" Traumatology	blood culture	NT	622/630	1	OX PC SM TE GM EM OL LI SP
8 /97	Hospital "E" Traumatology	pharynx	47/54 (III)	622	2	OX PC SM GM EM OL LI SP
9 /98	Hospital "F" Surgery	wound	NT	NT	5	OX PC TE GM EM OL LI SP
10 /98	Hospital "F" Surgery	wound*	NT	618/620/626/630	1	OX PC SM TE GM EM OL LI SP
11 /98	Hospital "F" Surgery	throat*	NT	618/620/626/630	1/1	OX PC SM TE GM EM OL LI SP
12 /98	Hospital "F" Surgery	nose*	NT	618/620/626/630	1	OX PC SM TE GM EM OL LI SP

Which should be the international standard typing method for the MRSA isolates? That was the question of Weller [41] who listed the advantages of different phenotypings such as antibiogram, phage typing, serotyping, whole cell protein analysis, immunoblotting, multilocus enzyme electrophoresis (MLEE), zimotyping and genotypings including: plasmid analysis, restriction enzyme analysis (REA), ribotyping, insertion sequences (IS), *mecA*: Tn554 probotyping, binary typing, pulsed-field gel electrophoresis (PFGE), PCR typing: coagulase gene typing, protein A gene typing, random amplified polymorphic DNA (RAPD), arbitrarily primed-PCR (AP-PCR), repetitive element sequence based PCR (rep PCR).

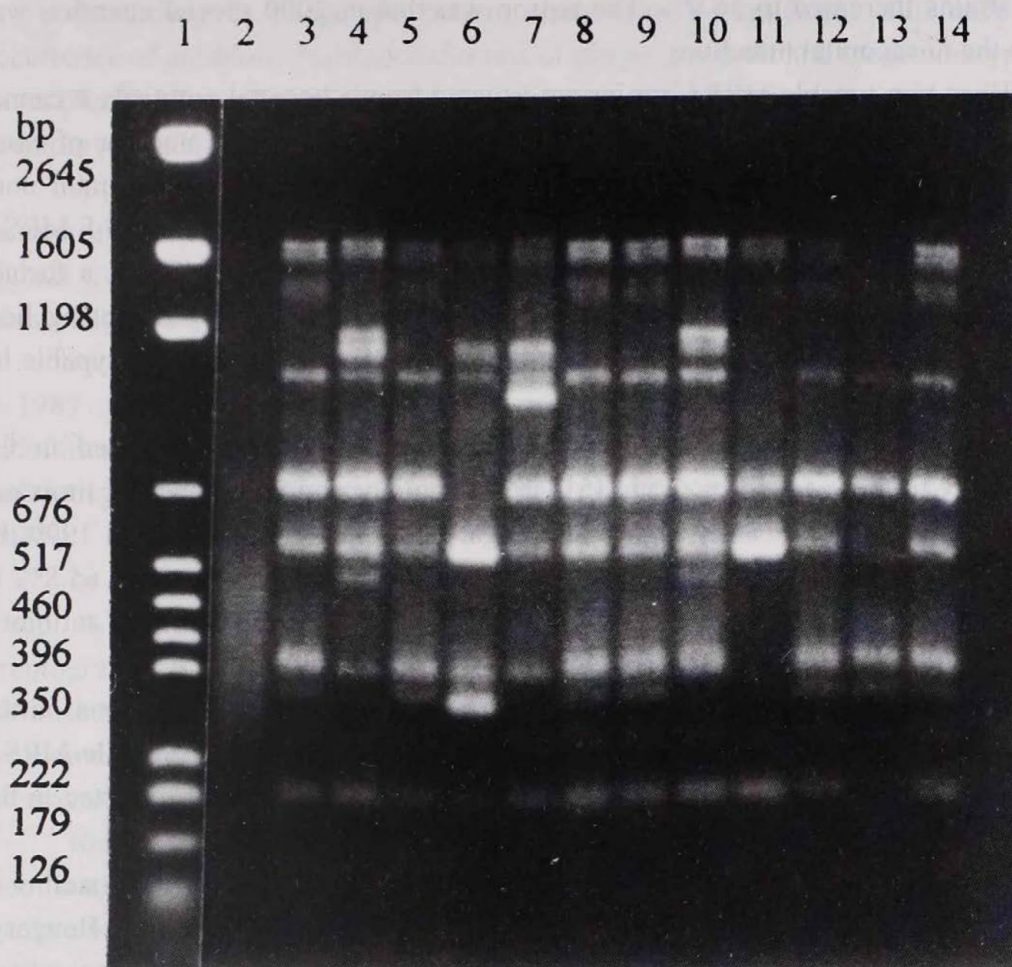


Fig. 7. AP-PCR patterns for 12 MRSA strains used in this study

1: markers, 2: negative control, 3–14: From left to right strains are numbered as in Table IV from 1/97 to 12/98

The conclusion was that only the phage method has been standardized for international use, but owing to the high proportion of non-typable isolates and poor reproducibility it should be completed by the "gold standard" PFGE method, which is highly discriminatory, but time consuming, and the international standardization is missing. According to Weller only the binary typing (based on Southern hybridisation) can be used as a single method.

In the present work 3391 *S. aureus* strains isolated between 1997 and 2000 were examined for phagetype-pattern and antibiotic sensitivity. 27.3% and 8.8% of strains originating from 1997 and 1998 proved to be MRSA, respectively. In 2000 the rate of

MRSA strains increased to 36.0%. The reason was that in 2000 special attention was given to the nosocomial infections.

When non-typable MRSA strains are isolated from a hospital outbreak, it cannot be stated which of the strains are responsible for the outbreak. In a number of cases high proportion of MRSA isolates originating from hospital outbreaks remain non-typable [48]. In England and Wales e.g. in the framework of a survey of MRSA infections carried out in 1990 only 3.8% of isolates were typable [49]. In a former examination 46.3% of our non-typable *S. aureus* strains became typable using heat treatment [50]. In the present work the non-typable MRSA strains became typable by basic phages in 56.7% using heat treatment.

Comparing the antibiotic resistance rates of MRSA strains obtained in the present study to those found by Witte [51] in the same period in Germany it turns out that the rates were significantly higher in Hungary than in Germany (e.g. in 1999 the MRSA strains showed tetracycline resistance in 16.0% in Germany, and in 65.8% in Hungary). The reason for this significant difference can rather be the abuse of antibiotic therapy than the spreading of different clones.

Multiresistance of MSSA and MRSA strains suited to the expectations: in the whole period of examination MSSA strains were multiresistant in 1.3% while MRSA strains in 83.5%. The difference in the occurrence of multiresistance is reflected in the effectiveness of phage-typing.

Witte gave an account about a narrower resistance pattern of MRSA strains in Germany after 1995 [51]. MRSA strains showed similar changes in Hungary: multiresistance decreased gradually from 1997 to 2000. The decrease was connected with lower tetracycline, streptomycin and gentamicin resistance rates, presumably due to the altered therapy with new antibiotics.

The significance of resistance plasmids was emphasized convincingly by Lacey [52]: "The rapid diversification in the properties of the staphylococcus over the last 20 years has resulted chiefly from alterations in plasmid carriage, by transfer of plasmids and phages between cells." In Hungary Jánosi [25] published data about different *S. aureus* phage patterns and plasmid profiles. Plasmids (3.7 kb, 2.9 kb and 1.25 kb) coding chloramphenicol, tetracycline, streptomycin and gentamicin resistances occurred in the 83A phage type complex. Rosdahl [53] investigated *S. aureus* strains isolated from hospitalized patients in Denmark for antibiotic resistance and phage-type in 1996, 1997, 1998 and 1999 and found methicillin resistant strains in 4.4%, 4.6%, 4.5% and 4.1%, respectively; gentamicin resistance in less than 0.7%, erythromycin resistance in less than 3.9%. The reason for the low rates of the antibiotic resistances was the applied antibiotic policy.

According to phagetype pattern there was a significant difference in the occurrence of antibiotic resistance. Strains of phage group III and those non typable by international basic phages showed the highest resistance to tetracycline, gentamicin, streptomycin and erythromycin in every year of observation.

An increased use of erythromycin in the recent years has led to an increasing resistance to erythromycin, especially in MRSA strains [51]. In the present study the highest frequency (93.4%) of erythromycin resistance occurred among MRSA strains which originated from nosocomial infections in the year 2000.

According to the large-scale examinations of János [25] performed from 1978 to 1989 with more than 10 thousand *S. aureus* strains, 15% were resistant to MLS antibiotics. In the present work from 1997 to 2000 MSSA strains were resistant to MLS antibiotics between 3.8% and 6.5%, while the MRSA strains between 75.6% and 93.4%.

In the former examinations in Hungary [28] the constitutive MLSR strains group A were the most frequent phenotypes. The PMSR (partial macrolide-streptogramin B resistant) phenotype (group B) strains were frequent in 1980–81 and diminished thereafter, group C1 and C2 diminished after a short ascending period. In our examinations in 1997 to 1999 with comparatively a fewer number of MRSA strains the macrolide type A occurred in 91.8%, the groups B, C1 and C2 were rare.

Resistance to macrolides in bacteria evolves through three mechanisms: (1) modification of the antibiotic target mediated by the *erm* gene; (2) enhanced efflux, mediated by the *msr*, *erp*, *mef* and *mre* genes; (3) inactivation of antibiotics by *ere* or *ere*-like and other inactivating enzymes. Sutcliffe et al. [36] and Schmitz et al. [54] examined the macrolide resistance genes of *S. aureus*. On the basis of their results we searched for *ermA*, *ermB*, and *msrA* genes of MRSA strains. We found *ermA* and, *ermA*+*C* genes in MRSA strains; *ermA* and *ermC* genes in MSSA strains and *ermA*, *ermC* and *msrA* genes in CNS strains. Unknown (not identified) genes were detected in MSSA and CNS in 1.8%.

In MSSA and MRSA strains originating from German hospitals [54] *ermA* genes were found in 21% and 40.3%, *ermC* genes in 46.5% and 35.8%, and unknown genes in 12.0% and 10.4%, respectively.

Since in Hungary there was an increase of infections caused by MRSA strains, and the phage typing methods – effective to MSSA strains – gave unsatisfactory results for the typing of MRSA strains we completed the phage typing method with the AP-PCR typing based on the observations of van Belkum [55] who made a comparison between phage typing and AP-PCR typing with satisfactory results. In a former cooperation we supplemented MRSA phage typing with PFGE typing [56] with

acceptable results. Our examinations concerning AP-PCR typing of nosocomial MRSA strains gave suitable typing results of the strains non typable by phages.

In this publication we give a review on our present possibilities concerning MRSA typing. It is hoped that international efforts will result in a generally accepted DNA based typing method, the need for which was emphasized recently also by van Belkum [57].

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PATHOGENESIS, MICROBIOLOGICAL AND CLINICAL ASPECTS OF ORAL CANDIDIASIS (CANDIDOSIS)

(A REVIEW)*

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The clinical significance of the oral candidiasis (either as independent disorder, or as a part of another disease) is increasing with time.

The diagnosis and local treatment of the oral candidiasis may not be satisfactory, this disorder cannot be eliminated without the correct diagnosis and management of the underlying disease. At the same time, some disorders, such as *Candida* induced leukoplakia, may significantly enhance tumor development.

Fungal infection of the mouth is often the initial sign of several immunodeficiency diseases. It is, therefore, very important to clarify the background of a fungal infection, since this may be critical regarding the prognosis.

Keywords: *Candida albicans*, oral candidiasis, adhesion of the *Candida albicans*

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Fungi occurring in the oral cavity

The fungi are achlorophyllous heterotrophic eukaryotes, which can reproduce only by using organic matter. The fungi present in the mouth are either saprophytic or facultative pathogens. Morphologically, filamentous, dimorphous fungi and yeasts are distinguished. Since fungi are much larger than bacteria, the structure of the former has been described by Schönlein and Gruby as early as in the nineteenth century.

Of the above types that occur in the oral cavity mostly yeasts can be detected. Among them about 150 candida spp. may be present, 80% of them *C. albicans*, *C. tropicalis*, and *C. glabrata*, and 20% other species, such as *C. parapsilosis*, *C. stellatoidea*, *C. guilliermondii*, *C. krusei*, *C. pseudotropicalis*, etc. The microbiological identification may be important, since during empirical treatment the pathogenic fungus may be *ab ovo* resistant to the antimycotic drug used, or resistance may develop during the treatment thereby the resistant fungi can be selected and become predominant. In such a case, both the correct identification of the strain and the determination of antimycoticum-sensitivity are of utmost importance [1].

Methods of the microbiological diagnosis

Simple and frequently used techniques are: microscopic examination of the matter to be studied using stained preparations, well as culture in a special medium. Identification is based upon morphological and biochemical characteristics. During microscopic examination the morphological properties of fungal cells are studied, such as the size, form, location and affinity for dyes of the cells. In native preparations the reproductive and metabolic activities can also be observed.

Culture is the *sine qua non* for the exact identification and determination of the antimycoticum-sensitivity of fungi. Using specific media, e.g. selective Sabouraud agar, fungi can be easily isolated from the specimen. For the identification of isolated fungi, and their antimycoticum-sensitivity, rapid tests and automatic identification techniques are available. These are based upon biomechanical characteristics, and provide results within 48–72h.

Using serological methods fungal antigens can also detected, even directly from the clinical specimen.

Candida albicans and candidiasis

Candida albicans can reproduce in different morphological types (budding or filamentous form). It is generally believed that the budding type is invasive and pathogenic, whilst the yeast cells are nonpathogenic. Data from the literature are, however, contradictory in this respect. Hyphae could not be found in some specimens taken from candida-infected tissues. Cultures of *Candida albicans* strains often undergo variations if the alimentary conditions are not optimal. At normal body temperature the budding forms occur frequently. The structure of the cells may also change, sometimes the translocation of chromosomes is seen [2, 3, 4]. In any case, the rate of reproduction plays a significant part in the pathogenicity.

Colonization and infection

The adhesion of the candida cells to host cells is necessary for colonization and it contributes to the persistence in the host. Without adhesion, the rate of multiplication of the fungus is not enough to maintain carriage of candida organisms in the oral cavity and gastrointestinal tract. The persistent adhesion is also important for providing the transition from colonization into infection. The change in the balance between colonization and infection may lead to the predominance of the latter by the alteration of expression of adhesion ligands and receptors in some patients [5, 6]. Candida cells are bound to several host cells including epithelial, endothelial cells and phagocytes. The mechanisms of adhesion are summarized in Table I [7–11].

From biophysical aspect, the nonspecific relationship between the markers of mucous membrane and fungi can be described as a hydrophobic-hydrophobic interaction. Biochemically, the binding proteins present on the surface of candida organism bind the glycoproteins located on the mucosa surface; this is another way of adhesion [12].

In case of a specific adhesin-receptor interaction, the adhesion - protein present on the surface of the candida cells is bound to the receptor-protein of the mucosa. The adhesins are named according to the protein to which they are bound, e.g. fibronectin-binding protein, fibrinogen-binding protein, thrombin-binding protein, etc. The more binding proteins a candida strain has, the higher is its virulence. *Candida albicans* expresses adhesins which recognize the extracellular matrix proteins including laminin, fibronectin and entactin. This yeast also has surface proteins that are similar to the mammalian integrin, such as $\alpha M\beta_2$, $\alpha\gamma\beta_2$, $\alpha_5\beta_1$. These are bound to endothelial receptors, e.g. ic3b and fibronectin. The lectin-like adhesins include surface proteins that are bound to fucosyl or N-acetyl glycosamine determinants or to galactosides on

epithelial cells. The adhesion of candida species to surfaces covered with saliva (e.g. dental prosthesis) may be of particular importance [10, 14].

Table I

Mechanisms of adhesion of C. albicans

Mechanism of adhesion	Adhesins of <i>C. albicans</i>	Receptors or mediators	Host (surface)
Hydrophobicity	Surface proteins	Hydrophobic surfaces	Epithelial cells, materials used in dentistry
	Integrin-like surface proteins e.g. $\alpha M\beta_2$ $\alpha\chi\beta_2$ $\alpha\beta_1$	Ic3b, c3d, polypeptides (e.g. fibronectin)	Epithelium, endothelium, extracellular matrix, blood
Protein-protein binding	Surface proteins	C3d, extracellular matrix proteins (e.g. fibrinogen, collagen, laminin, fibronectin, entactin)	Epithelial cells, endothelial cells
	Surface proteins	Fucose or N-acetylglucosamine residues on the host glycoproteins	Epithelial cells
Lectin-like attachment	Surface proteins	Streptococcal polysaccharides	Colonized epithelial cells, dental plaque
	Mannoproteins	Glycosphingolipid receptors	Epithelial cells

The surface structure of *Candida albicans* can vary. The expression of surface macromolecules may be changed by environmental factors. These changes enable the candida organism to escape from the immune defense or to prevent the adherence to other cells, thereby promoting candidiasis. The change in glycosilation of surface proteins may give hydrophobic character to a protein, thereby the adhesive capacity may be increased. The interaction of the yeast and host cells may also be influenced by exogenous factors, e.g. drug treatment. Antibiotics, by killing competitive

microorganisms, can make new sites available for candida colonization. By contrast, the antifungal agents may reduce the adhesion [15, 16].

In case of a superficial injury, such as a decubitus ulcer caused by a denture, some receptor proteins (fibrinogen, fibronectin, thrombin) may appear on the mucosal surface. If this process reaches deeper layers, then collagen, vitronectin or laminin may also be exposed. Adhesin proteins will be attached through adequate binding proteins to the above molecules which gives rise to biofilm formation and subsequent growth of fungi. In such a way candida species can mask itself by body's markers, thus the host will recognize it as his own macromolecule. As a result, the yeast will not be available for the potentially compromised immune system. During fungal colonization on the mucosal surface hydrophobic-hydrophobic interaction takes place, and the yeast cells coalesce. Thus their virulence will be increased since they cover one another [12, 17].

Extracellular (excretory) virulence factors

The excreted hydrolytic enzymes often play a significant role in the pathogenesis of microorganisms-induced diseases. *Candida albicans* can secrete several enzymes, such as lipase, phospholipase, phosphomonoesterase, hexoseaminidase, α glukosidase, proteinases [18, 19]. Mostly these latter were studied, but the final conclusion is still missing: whether extracellular protease activity is always accompanied by infection or not [20]. In addition to proteases, other factors may also contribute to the infection and the development of disease [21, 22]. Fungal toxins show versatile activities, namely cytotoxic, pharmacological, immunological, enzymatic, shock evoking and infection enhancing activities [1, 23, 24].

Defensive mechanisms of the host

Microbial interactions

The normal flora of the mouth, in addition to bacteria, also contains different fungal strains that are bound by a sialoprotein. Thus, an equilibrium can develop, and, under physiological conditions, neither bacteria nor fungi can become predominant [25, 26].

The microorganisms, present in the oral cavity compete with one another for the binding sites located on epithelial cells. In equilibrium, this prevents a massive colonization of candida sp. [27, 28]. If an obligate pathogenic microorganism, e.g. *Staphylococcus aureus*, and a candida sp. coexist, the bacterium may occupy the binding sites on epithelial cells (e.g. displacing other microbes) which may break up the balance and lead to angular cheilitis [29, 30, 31].

Humoral defense

Non-immune factors in the saliva

Iron

The elevation of iron level in the plasma and saliva will increase the susceptibility to infections because iron as a nutrient is critically important for the growth/reproduction of fungi and bacteria [32, 33].

Lysozime (muramidase)

This enzyme acts as antimycotic in several ways. It partly impairs cytoplasmic membrane through increasing permeability and hydrolyses cell wall structures thereby damaging candida cells, partly (together with IgA antibodies) stimulates phagocytosis [34, 35].

Histidine-rich polypeptides (HRPs)

These salivary histidine-rich proteins potentiate the effect of lysozime which results in stronger antifungal activity [36, 37].

Lactoferrin

The concentration of lactoferrin in saliva significantly increases during inflammation of the mucosa and parotid gland. This protein binds iron thereby reducing salivary iron concentration which, in turn, will produce antifungal activity. It is more effective in the apolactoferrin form because its iron saturation level is lower, thus it can bind more iron. The sensitivities of different candida spp. both to lysozime and to lactoferrin vary significantly [38, 39, 40].

Lactoperoxidase

The antimicrobial effect of lactoperoxidase is also achieved through several mechanisms [41]. These include halogenization of microbial proteins, formation of aldehyde and oxidation of SH groups of thioamine-lipids [42]. Significant candidacidal activity was observed when candida spp. were incubated with a conjugate of lactoperoxidase, xanthine oxidase and specific antibodies against *Candida albicans* [43].

The salivary *glycoproteins*, that are similar to blood group antigens, appear on the mucosal surface and increase the buffer capacity of the saliva.

Salivary immune factors

The most important specific immune factor in saliva is the secretory immunoglobulin A (sIgA). This globulin represents the primary humoral specific defense against oral candida infection. It aggregates the yeast cells thereby preventing their adherence to the mucosal epithelium, and opsonizes them which facilitates phagocytosis.

Cellular defense

Extraorally several cellular and noncellular components of the immune system take part in the elimination of fungal infections. The defensive system includes neutrophil granulocytes, one of which can phagocytose up to ten yeast cells and kill a part of them mostly through the activity of myeloperoxidase [44, 45].

The fungicidal effect of human neutrophils is enhanced by interferon- α and tumor necrosis factor. Of the cytokines the granulocyte colony-stimulating factor stimulates the formation of granulocytes in the bone marrow [46, 47].

The candida cells are eliminated mostly by phagocytosis which can be achieved with the participation of the T-cell-produced cytokines/lymphokines [48, 49].

Infection and disease develop if a) candida organisms can produce pathogenicity and virulence factors continuously and in large amounts, b) adhesion and colonization cannot be prevented by the host for any reason. As a result, candida-biofilm is formed under which the extracellular enzymes and toxins of the yeast damage the tissues continuously thereby being ready to produce systemic infection.

In summary, the pathogenesis of oral candidiasis is rather complex: in addition to the microbiological properties of candida spp., the actual status of the host also plays a significant role.

Factors influencing candida carriage

It is generally accepted that, including healthy population, the candida carriage in women somewhat exceeds that seen in men. This is thought to be due to the extensive use of oral anticoncipients [50]. Yeast carriage appears to be more frequent in summer which may be explained by the change in circulatory regulation. It is not clear whether temperature influences candida-virulence. It has been established, however, that at lower temperature yeast cells are more resistant to the killing effect of leukocytes.

Salivary factors

Reduction in the amount of saliva leads to the decrease in the buffer capacity of the saliva. Saliva contains some proteins (lysozyme, lactoferrin) and histidine which have antifungal activity. If the volume of saliva becomes less, the antimycotic effect will also be reduced which in turn results in the increased rate of reproduction of the oral candida strains [29]. Decrease in the salivary pH also increases the number of candida organisms. The saliva freshly secreted by the parotid gland is more resistant to the candida-produced proteases than the saliva mixed with other materials [51].

Diurnal variations

During sleep, when salivary secretion is reduced, there is more chance for candida carriage. Sleeping with dentures also increases yeast carriage since the hydrophobicity of denture material is greater than that of mucosa. Thus, the adherence of the yeast to the denture will increase, and during sleep with dentures the number of fungi will be significantly higher than during sleep without dentures [52, 53].

Smoking

Some authors reported that smoking increases the number of candida organisms by 30–70%. This may be due to localized epithelial changes, and to the fact that some substances present in cigarette smoke (such as polycyclic aromatic hydrocarbons) provide aliment, primarily carbon, to the yeast; they also serve a source of energy to other candida species. Thus, smoking accelerates the reproduction of candida organisms [21].

Distribution in oral cavity

There are some distinguished sites in oral cavity that are particularly important for candida colonization. Such regions include the posterior dorsal part of the tongue and the bony palate where the proliferation of candidal hyphae is obvious. In persons with dentures, primarily the fitting surface serves as a reservoir for the yeasts. The inner buccal surface and corners of the mouth are also preferential sites for adherence and reproduction [52, 54–56].

Immune status

Candida carriage was found to be greater in persons with blood group 0 or in whom the blood group antigens are not secreted in saliva. Candida carriage is also modified by the amounts of specific antibodies (IgA, IgG) against *Candida albicans* as well as by reduced ratio of T_{helper} to T_{suppressor} lymphocytes [57].

Oral microflora

Several microorganisms can be isolated from the normal oral cavity whose quantitative and qualitative distribution can produce a normal balance in microflora. This balance may be broken by several factors. The loss of balance, however, does not necessarily mean candida infection, but it may facilitate adhesion, multiplication and finally the predominance of the yeast. The causative factors include use of broad spectrum antibiotics, corticosteroids or oral anticoncipients as well as overuse of solutions for mouth irrigation [25]. Oral sex can also change the normal flora significantly. The composition of the oral microflora may be altered unnoticeably by diets containing broad spectrum antibiotics. Some of these are admixed to animal food to increase meat production. Such drugs include tetracyclines, aminoglycosides and macrolides. In some countries, fluoroquinolons are used to eradicate salmonella infection in pigs. The regular consumption of the meat of these animals provide a chance for the overgrowth of resistant bacteria.

Hospitalization

Hospitalization may also be a factor modifying candida carriage. The oral carriage of yeast is significantly higher in hospitalized than ambulant patients. The high counts alone, however, do not necessarily mean that the patient will have a manifest candidiasis [58]. The factors promoting or modifying oral candidiasis are summarized in Table II.

Clinical types of oral candidiasis

Oral candidiasis can be classified clinically from several aspects. Classification is based upon the clinical manifestation of fungal infection, histopathological alterations and the potential presence of an underlying disease. The clinical manifestation of candida infection may vary considerably. There may be overlaps of the individual diseases; their differential diagnosis and classification are often difficult.

Pseudomembranous candidiasis

This is the most common form of oral candida infections. It is characterized by white patches which can be wiped off from the surface of the tongue or the mucosa.

Underneath an erythematous base with punctate bleeding is seen. This form of candidiasis is observed mostly in infants and elderly patients. In the former group, the immune system, due to its underdevelopment, is not yet able to eliminate the fungus, and in the elderly the immune defense is not able to do so for any longer.

Table II*Factors influencing oral candidiasis*

Exogenous factors	
	Chronic local irritation
	Yeast content of food
	Alimentary factors
	Antibiotic and/or hormone content of food
	Radiotherapy of head and neck regions
	Age
	Hospitalization
	Smoking
Dentures	
	Ill-fitting denture
	Improper use of denture
	Oral hygiene
Endogenous factors	
	Change in oral microflora
	Immunologic and endocrine disorders
	Malignant and chronic diseases
	Haemopoietic disorders, blood group
	Dysplasia of oral epithelium

Treatments with immunosuppressive agents, used to manage underlying diseases, diminish the natural defence of the body. In these cases, one should pay more attention to the potential candida infection and be ready to introduce antifungal prophylaxis. The pseudomembranous candidiasis is also common in the initial stage of Human Immunodeficiency Virus (HIV) infection. Candidiasis may be the first premonitory sign of this viral disease. The removable white patches seen in pseudomembranous candidiasis comprise necrotic tissue and desquamative parakeratotic epithelium. Under this, on the outer surface of epithelium, microabscesses containing polymorphonuclear neutrophils and oedema can be observed. The candida cells and hyphae reach the stratum spinosum [27].

Erythematous (atrophic) candidiasis

This form is primarily seen on the dorsum of the tongue, palate and buccal mucosa. The patient complains of burning, sharp sensation which is due to the loss of papillae on the dorsum of the tongue. Previous corticosteroid and tetracycline treatments are thought to be predisposing factors. Erythematous (atrophic) candidiasis may follow persisting pseudomembranous candidiasis, but it can also develop "de novo". Red areas are often seen in the palate which result from increased vascularization and reduced epithelial thickness [59, 60].

Candida leukoplakia (hyperplastic candidiasis)

Candida leukoplakia is a white patch (<5 mm in diameter) firmly attached to the oral mucosa. Bánóczy et al. have reported [54, 61–64] that, in many cases, the histologically diagnosed leukoplakia is not white. The histologic characteristics include parakeratosis and epithelial hyperplasia. The malignant transformation depends on the degree of dysplasia. It is still unclear, whether *Candida albicans* is a causative factor of leukoplakia, or this disorder is the consequence of a secondary infection. It has been corroborated that this group of leukoplakia, the so called nonhomogeneous form, is a severe disorder which is prone to malignant transformation. While the malignancy rate amounts to 2–6% for all kinds of leukoplakia, this ratio for the nonhomogeneous form is 9–40%. It is therefore very important to concentrate the treatment on the candida infection in the case of candida leukoplakia, and to try to change the nonhomogeneous into the homogeneous form [65–68].

Candida-associated denture stomatitis

This disorder is primarily observed in patients with upper dentures. Erythematous and oedematous areas are seen in the mucosa which alteration contacts the fitting surface of the denture. Otherwise the patients appear to be completely healthy, no pathological symptoms or signs are found in their history. The pH of saliva between the denture and mucosa is lower than in normal persons; this is favourable for candida reproduction. Yeasts could be detected in 78–100% of patients with denture stomatitis, but this does not necessarily mean that these patients will have manifest candidiasis. The yeast count in persons with dentures is ten times higher than in those without dentures. In addition, sleeping with denture also increases candida count. Newton [69] distinguished three clinical types of candida-associated denture stomatitis:

- I. hyperaemic
- II. erythematous
- III. granular

Denture stomatitis is not associated exclusively with candidiasis. Bacterial infection, mechanical irritation and allergic reaction may also play a part in stomatitis [53, 70].

Angular stomatitis

Angular stomatitis is an uni- or bilateral disorder of the angles of the mouth which is characterized by inflamed and erythematous fissuring. It may be ulcerated in severe cases. Inducing factors include B₁₂- or iron-deficiency, but the disease may accompany HIV infection. Angular stomatitis is induced not only by fungi, but also by *Staphylococcus aureus*, which reproduces as a result of synergism of pathogenic microorganisms. The disease often associates with denture stomatitis, but it may be caused by saliva accumulation resulting from the lowering of the physical biting height. It may also accompany other underlying diseases such as chronic multifocal oral candidiasis [31, 55].

Median rhomboid glossitis

This disorder is characterized by an atrophic lesion which is elliptical or rhomboid in shape, symmetrically located at the midline of the tongue. Usually, there is a chronic candidiasis in the background, and manifested primarily by smoking and diabetes mellitus. The gustatory buds are atrophic, the hyphae infiltrate the upper layer of parakeratotic epithelium and form a biofilm. Usually, a mixed flora (bacteria and fungi) can be isolated [59].

Chronic multifocal oral candidiasis

This disorder is a group of lesions in multiple oral sites, usually appearing during a prevailing chronic candida infection. This disease comprises a tetrad that include a) leukoplakia [14], the most constant component of the tetrad, b) angular cheilitis [61], especially in denture wearers, c) median rhomboid glossitis [54], d) palatal lesions.

Holmstrup and Bessermann proposed additional criteria: the lesion lasts longer than one month and no predisposing factors are found in the medical history. The patients who underwent radiotherapy or received antibiotic or cytostatic treatment are excluded from the above syndrome [71].

Pindborg reported that this disease occurs most commonly in smoking men aging 50–60 years. The successful antifungal treatment and the prevention of recurrence necessitate the reduction or stop of smoking [68].

Oral candidiasis associated with systemic disease

Oral candidiasis is often a manifestation of some systemic disease. This can be an immunological and/or haematological disorder which may lead to candida infection by compromising the defensive system of the body. The symptoms of systemic diseases are present not only in the oral cavity but also in horny tissues (e.g. skin, nail) and on the mucosal surface (e.g. vagina, intestines) [31].

Treatment of candidiasis

The antifungal agents used for treating fungal infections are listed in Table III. It is crucially important to treat the potential underlying disease together with the mycosis, otherwise the recurrence will be inevitable. In each case, the dentist taking part in the diagnosis and treatment of oral candidiasis should cooperate with the patient's doctor to find the adequate therapy. Because of the different antifungal-sensitivity of candida species, it is reasonable to obtain a specimen before starting the treatment and send it to a laboratory in order to determine the antimycotic sensitivity. In case of unsuccessful treatment, it is compulsory to stop the previously used medicine and switch to the effective antifungal agent [12, 72–76]; see Table III.

Table III*Antimycotics used for the treatment of candidiasis*

Polyenes (bind irreversibly to the ergosterol of fungal cells)	
	Nystatin
	Amphotericin-B
Azole-derivatives (inhibit ergosterol synthesis)	
	Clotrimazole
	Miconazole
	Ketoconazole
	Fluconazole
	Itraconazole
DNA-analogues (inhibit protein synthesis of fungi at DNA-level)	
	5-Fluorocytosine

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CHRONIC INFECTIONS AND ATHEROSCLEROSIS*

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The inability of traditional risk factors such as hypercholesterolemia, hypertension, and smoking to explain the incidence of atherosclerosis (AT) in about 50% of the cases prompted a search for additional putative risk factors involved in the development of the disease. Infectious agents have long been suspected to initiate/contribute to the process of AT. It has also been suggested that inflammation, either related to infectious agents or independent from infection, may mediate the atherogenic process [1, 2].

Keywords: atherosclerosis, *Chlamydia pneumoniae*, Human cytomegalovirus

In vivo evidence pointing to the involvement of infectious agents in the AT process was first demonstrated in the chicken model. In both normocholesterolemic and hypercholesterolemic chickens, typical AT lesions were caused by infection with an avian herpesvirus, the Marek disease herpes virus (MDHV). Analysis of the arterial tissues further revealed increased levels of total aortic lipids, especially cholesterol, cholesteryl esters and phospholipids, suggesting that the cholesterol uptake of cells in the arterial wall is induced by *in vivo* MDHV infection. Importantly, the atherogenic effect of the virus was shown to be preventable by the use of a herpesvirus vaccine [3–5]. The involvement of infectious agents in the process of human AT was suggested by the detection of the presence of various infectious agents in human AT lesions by seroepidemiological data indicating higher rates of seropositivity to these agents in AT

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patients versus a control population and by animal studies in which AT lesions similar to human AT lesions were induced in animals infected with human pathogens. The most strongly implicated infectious agents are *Chlamydia pneumoniae* (*C. pneumoniae*), an obligate intracellular bacterium that causes acute and chronic respiratory diseases, and human cytomegalovirus (HCMV), an ubiquitous herpesvirus, that often infects humans very early in life and establishes latency in the infected host. Other pathogens such as *Helicobacter pylori* (*H. pylori*) and herpes simplex virus (HSV) have also been suggested as agents contributing to the development of the disease [6–10].

The presence of pathogens in AT lesions

DNA and/or proteins of various pathogens, including *C. pneumoniae*, HCMV and HSV, have been shown to be present in human AT plaques by polymerase chain reaction (PCR) or immunohistochemical techniques. It is noteworthy that, whereas *C. pneumoniae* was not present (or only very rarely) in non-atheromatous tissues, HCMV and HSV DNA were also detected in normal areas of the vessel walls [11–13]. More importantly, *C. pneumoniae* was cultured from the coronary arteries of a patient with coronary AT indicating the presence of live bacteria in the diseased tissues [14]. The cells in the AT lesions that harbor the DNA of these pathogens have not been clearly identified. It is possible that the pathogens infect endothelial and smooth muscle cells of the arterial walls and reside in these cells in a non-replicating form, but these pathogens may well also be present in the monocytes/macrophages and lymphocytes that are the components of the AT lesions. Monocytes are not fully susceptible to HCMV replication, e.g. viral DNA is present in these cells, but viral gene expression occurs only when the monocytes are differentiated into macrophages [15–17]. Differentiation of monocytes may proceed into macrophages at the site of vascular injury, e.g. in the course of coronary angioplastic surgery, leading to the expression of immediate early (IE) or other gene products of HCMV, which then exert their effects on the atherogenic process. Indeed, the expression of IE antigens has been observed in cells of the coronary arteries following angioplastic surgery, suggesting an association with restenosis, an accelerated form of AT, and the reactivation of HCMV-IE antigens in the arterial wall [18]. *C. pneumoniae* DNA has been detected by PCR in the aorta, coronary arteries, carotid artery and arteries of the lower extremities [12], and additionally in the middle cerebral arteries, important sites of cerebral thrombosis [19], indicating that cerebrovascular diseases, including stroke, may also be related to infection of the arterial wall with *C. pneumoniae*. Our results showed that *C. pneumoniae* DNA was present in 5 of 15 AT samples of middle cerebral arteries, but not in control, non-atheromatous tissues (Fig. 1). Transmission electronmicroscopy of

PCR-positive samples revealed the presence of *C. pneumoniae*-like bacterial structures, confirming the PCR results [19].

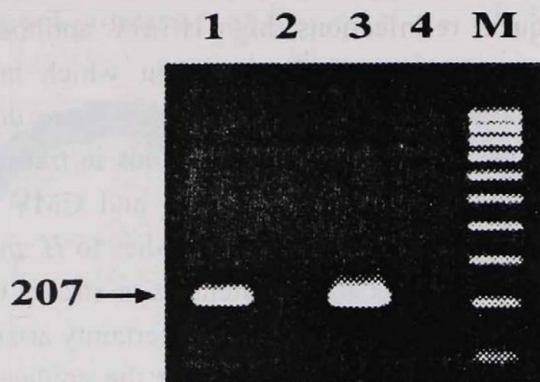


Fig. 1. Nested PCR (nPCR) amplification of *C. pneumoniae* ompA gene

Lane 1, nPCR-positive atherosclerotic middle cerebral artery sample (207 bp fragment) amplified as described [19, 72]; lane 2, nPCR-negative control cerebral artery sample; lane 3, nPCR-positive control prepared from *C. pneumoniae* infected McCoy cells; lane 4, nPCR-negative control prepared from uninfected McCoy cells; lane 5, molecular size marker (100 bp ladder; Sigma, St. Louis, MO)

Association demonstrated by seroepidemiological studies

Saikku et al. [20] revealed an association between serum antibodies to *C. pneumoniae* and myocardial infarction and other forms of coronary artery disease. In the past 12 years, research groups in various countries have reported similar findings concerning coronary artery disease, myocardial infarction and AT disease in other areas of the vascular system, including the aorta, the carotis and arteries in the brain and lower extremities [21]. A few reports have also been published which did not confirm a serological association between AT and *C. pneumoniae*, probably because of the old age of the patient and control subjects with high background level of *C. pneumoniae* antibodies, or an ongoing *C. pneumoniae* epidemic in the study population [21]. In Hungary, elevated levels of *C. pneumoniae* antibody have been reported in AT patients [22]. In our studies significantly higher levels of IgG antibodies specific to *C. pneumoniae* ($p=0.021$), but not to HCMV ($p=1.00$), have been documented in patients with coronary artery disease verified by coronarography versus control patients. The difference between cases and controls for *C. pneumoniae* antibodies remained significant after adjustment of the data for age and sex, with a relative risk factor (odds ratio) of 2.2 [23]. The association of the cell-mediated immune (CMI) response to *C.*

pneumoniae and AT disease has been poorly studied, probably because of the technical difficulties posed by the determination of *C. pneumoniae*-specific CMI, and especially cytotoxic T lymphocytes.

Elevated levels of HCMV-specific antibodies in AT patients have been observed in several studies [6, 24], suggesting that, as a result of a periodic reactivation of the latent virus, or frequent re-infections, high HCMV antibody levels might be associated with AT. However, most of the cases in which an association was demonstrated between the disease and HCMV antibodies were defined as coronary restenosis after atherectomy or the development of lesions in transplanted hearts, and the association between primary coronary artery AT and CMV antibodies is less convincing [8, 23, 25]. In some studies levels of antibodies to *H. pylori* were elevated in patients with myocardial infarction, coronary stenosis or stroke, but failure to make appropriate adjustment for potential confounders, or uncertainty arising in consequence of the small numbers of cases and controls, mean that the epidemiological evidence provided by these studies for or against a solid association can not be regarded as convincing [6, 9, 25].

Animal models

Since AT is a multifactorial disease with a long progression period, the Koch postulates are difficult to fulfil. Nevertheless, several publications have documented inflammatory lesions similar to early AT lesions in humans in the arterial walls of mice or rabbits infected with murine CMV (MCMV), a biologically and structurally related virus to HCMV, or *C. pneumoniae*, respectively. For example, MCMV induced such lesions in normocholesterolemic BALB/c mice and in mice fed with a cholesterol diet. The lesions were significantly larger and increased in number if the mice were mildly immunosuppressed by gamma-irradiation at the time of infection, indicating that prolonged infection is beneficial for the development of the disease [26]. The serum low density lipoprotein (LDL) level was also significantly higher in mice infected with MCMV, as compared with that in mice that were not infected, suggesting that the lipid metabolism in the MCMV-infected host is also impaired, which might also be a component in the development of the disease [26]. Likewise, we (Fig. 2) and others have shown that repeated *C. pneumoniae* infections induce inflammatory lesions and early AT plaques in mice and rabbits [27, 28]. Both *C. pneumoniae* and MCMV infection accelerate the progression of AT in apolipoprotein-E (apo-E)-deficient mice, which develop the disease spontaneously [29, 30]. In the rat transplantation model, AT development was faster in animals receiving aortic allografts if the animals were infected with rat CMV [31]. The degree of neointima thickening was increased in rat

CMV-infected rats after carotid balloon injury as compared with that in uninfected animals [32]. Thus, animal models comprise a difficult but very useful approach to the establishment of a strong relationship between infectious agents and the AT process. The results suggest that *C. pneumoniae* and CMV initiate an inflammatory process in the arteries, which may lead to the development of AT and these pathogens can also enhance an ongoing AT process.

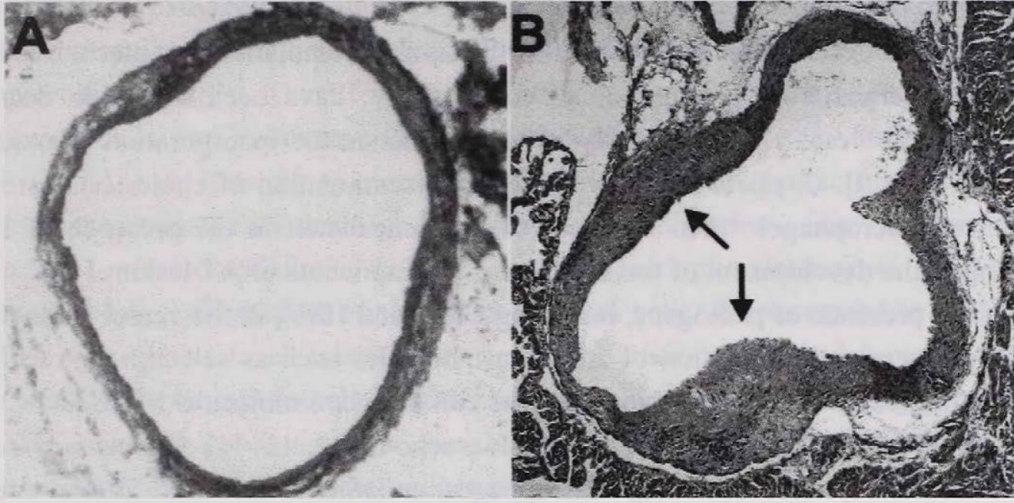


Fig. 2. Aortic changes following repeated *C. pneumoniae* inoculations of mice

Panel A: section of the aorta from an uninfected mouse (H&E, $\times 500$), note the smooth lining of the aorta;
Panel B: section of the aorta from a mouse 2 weeks after 4 *C. pneumoniae* inoculations (4 weeks apart) (H&E, $\times 500$), note the thickening of the intima and subendothelial inflammatory lesion (arrows)

Possible mechanisms of initiation/acceleration of the AT process by infectious agents

Mechanisms by which certain pathogens might be involved in the development of the AT process are based on A) the presence of these pathogens in the AT lesions leading to direct effects, or B) the hypotheses that indirect, systemic effects of the pathogens residing outside the vascular system are involved in the development of the disease, and the presence of the pathogens is not necessary in the vessel wall. Obviously, direct and indirect effects may also act in concert in the development of the disease.

A) Assuming the presence of the pathogens in the vessel walls, such direct effects include an increased mitotic activity and the proliferation of semipermissive and

permissive non-human and human cells, such as human vascular smooth muscle cells, following HCMV infection [33, 34]. One of the molecular mechanisms by which HCMV may lead to an increased cellular proliferation is the physical binding of the IE2 product of the virus to the tumor suppressor protein p53, thereby inhibiting its function [18, 35, 36] and *C. pneumoniae* infection [37] of cultured coronary artery smooth muscle and other cells inhibits apoptosis, leading to an accumulation of the infected cells, which may initiate vessel wall lesions or may increase the size of AT lesions.

Direct effects might be involved in the lipid accumulation by infected cells. A number of herpesviruses, including HSV and CMV, have been shown to decrease intracellular cholesteryl ester hydrolysis, or to enhance the incorporation of oxidized LDL [26, 38–40]. *C. pneumoniae* promotes the accumulation of cholesteryl esters in monocytes/macrophages when infected cells are incubated in the presence of LDL, resulting in the development of foam cells, early components of AT lesions [41].

The presence of pathogens, including CMV and HSV, in the vessel walls might induce an increased expression of adhesion molecules such as selectins, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) that are essential for leukocyte-endothelial cell interactions [32, 42–44]. *C. pneumoniae* has been found to replicate in human macrophages, endothelial cells and arterial smooth muscle cells [45] and to cause the upregulation of these adhesion molecules [46]. The stimulated expression of adhesion molecules enhances the adhesion of monocytes and lymphocytes to the vessel walls. These monocytes and lymphocytes then produce inflammatory cytokines that contribute to the development of the disease. Further, the infected endothelial cells change their normally anticoagulant functions into procoagulant functions with an elevated level of thrombin production and a decreased rate of thrombomodulin production. Importantly, altered endothelial cells produce molecules that are involved in the oxidation of LDL, further increasing the proatherosclerotic functions of these cells [47, 48].

Dendritic cells probably deserve special attention as regards the development of AT lesions since AT plaques are rich in dendritic cells [49] and since HCMV infects monocyte/dendritic cell precursors present in the bone marrow and the virus genome persists in these cells [17, 50] with possible reactivation on vascular injury. No data are available concerning the interaction between *C. pneumoniae* and dendritic cells. However, *C. trachomatis* and *C. psittaci*, species closely related to *C. pneumoniae*, induce the secretion of high levels of tumor necrosis factor- α (TNF- α) and the maturation of dendritic cells [51]. If similar effects are exerted on dendritic cells by *C. pneumoniae*, a further mechanism(s) could be suggested for the contribution of *C.*

pneumoniae to a disturbance within the plaque milieu, which might lead to thrombosis or other complications of AT.

B) Certain experimental data suggest that systemic effects of viral/bacterial infections contribute to the development of the disease, and the actual presence of the pathogen in the vascular wall might not be essential. Such indirect effects include elevated levels of circulating cytokines, acute phase reactants, humoral and/or CMI responses to a pathogen that interacts with cellular components of the vessel wall through molecular mimicry, and increased levels of human heat shock proteins (HSPs) and antibodies to HSPs. Thus, *C. pneumoniae* replicates in *in vitro* cultures of human endothelial cells and activates the production of monocyte chemotactic protein-1 (MCP-1) and IL-8. Relating to elevated levels of chemotactic proteins, a significantly increased transendothelial neutrophil and monocyte migration was observed in *C. pneumoniae*-infected endothelial cells [52, 53]. Interestingly, although *C. trachomatis* strains A and E replicated well in these endothelial cells, the upregulation of MCP-1 and IL-8 was unique to *C. pneumoniae*, and *C. trachomatis* strain L2 caused only a slight increase in transendothelial neutrophil migration. These results suggest that the role of *C. trachomatis* in the development of AT is less significant than that of *C. pneumoniae* [52]. Further, the adherent fraction of human peripheral blood mononuclear cells (PBMCs) cultured *in vitro* for at least 3 days, consisting of dendritic cells and monocytes/macrophages, replicated *C. pneumoniae* and secreted increased levels of TNF- α , IL-1b, IL-6 and interferon- α (IFN- α) [54]. The ability of *C. pneumoniae* to survive inside mononuclear cells suggests the importance of these cells *in vivo*. For example, *C. pneumoniae* could escape from host defense mechanisms and be transported from the infection site to remote target tissues, e.g. the vascular wall. The increased production of cytokines by these cells suggests a role of monocytes/macrophages in the development of the disease. However, it seems that the fate of *C. pneumoniae* is strongly dependent on the host cells, since infected monocytes carry *C. pneumoniae* inclusions at various developmental stages, whereas the development of the infective elementary bodies, the infectious forms of *C. pneumoniae*, is inhibited. On the other hand, macrophages support the full replication of *C. pneumoniae* [55]. Consistent with this observation, a highly differentiated human monocytic cell line, MonoMac, is susceptible to the full replication of *C. pneumoniae* and long-term infection can be established in these cells. Thus, monocytes/macrophages may contribute not only to the dissemination of *C. pneumoniae*, but also to host defense and immunoreactive mechanisms and to the maintenance of long-lasting local inflammation in the vessel walls [56]. However, a recent study which addressed the question of how the bacteria are transported from the

lungs to the cardiac vessels established that the cell population within the PBMCs that harbors *C. pneumoniae* DNA is predominantly the circulating CD3⁺ T cell population, and to a lesser extent the adherent population consisting of monocytes/macrophages and dendritic cells. The significance of these results remains to be determined, but they support the hypothesis that *C. pneumoniae* is transported from the lungs via the cellular route [57]. Concerning the humoral or CMI responses induced by antigenic peptides of a pathogen, cross-reactions with epitopes of the host cells causing immune injury of the host have been postulated. Similar mechanisms have been suggested for the development of a number of autoimmune diseases, such as diabetes mellitus and Guillain-Barré syndrome [58, 59]. Interestingly, antigenic mimicry between chlamydia peptides and cardiomyocytes is implicated in the development of cardiomyopathy [60].

Many data suggest the involvement of HSPs and antibodies to HSPs in the development of AT. For example AT lesions were induced by the immunization of rabbits with human HSP60 [61], which is induced in humans by various forms of stress, including hypertension, oxidized LDL and smoking. Bacterial HSPs display a high homology to human HSPs and may induce humoral and CMI responses that cross-react with host cell HSPs, causing endothelial or smooth muscle cell injuries in the vessel walls that lead to AT. However, no direct data are available to support the hypothesis that pathogens induce AT through molecular mimicry. In fact, our data concerning the interactions of coronary arterial diseases and antibodies to human HSP60 or *C. pneumoniae* suggest that antibodies to human HSP60 or *C. pneumoniae* are independent risk factors, and reveal that the simultaneous presence of both antibodies may result in a highly elevated joint effect promoting development of the disease (odds ratio: 82.0) [23]. Additional infection-related secreted factors, such as TNF- α , which induces a high expression of adhesion molecules in the endothelial cells, and elevated levels of matrix metalloproteinases, which degrade the connective tissue and weaken the fibrous cap on the atheromatous plaques, might also contribute to the development of AT, or to plaque instability and rupture, a usual cause of death from AT [62, 63]. Indeed, our results indicate elevated levels of IFN- γ and TNF- α in lung suspensions of mice inoculated intranasally with *C. pneumoniae* [64], suggesting that *C. pneumoniae* might exert an atherogenic effect through this mechanism. Elevated levels, and localization in the AT lesion, of C-reactive protein, an acute phase reactant and indicator of inflammation, are also suggested to be contributors to, or indicators of AT [65]. Other systemic effects include mechanisms based on the increased expression and release of various growth factors from fibroblasts and smooth muscle cells infected with HCMV. Some of these proteins have not been fully characterized [66], but certain CMV-induced growth factors have been characterized at the mRNA level as basic

fibroblast growth factor, acidic growth factor, platelet-derived growth factor (PDGF) or PDGF receptor [34].

Multiple pathogens in the development of AT

It should perhaps not be considered as a chance observation that a number of pathogens have been shown to be associated with the development of the disease by the seroepidemiological data and detection of the pathogens in the AT lesion. It is quite possible that many pathogens are able to exert an atherogenic effect by local infection of the vascular cells or by a systemic effect that involves the induction of inflammatory cytokines, acute phase reactants or some other mechanisms. Consecutive infections might be related to the progression of the disease, but if various pathogens are present in the arterial wall at the same time, molecular interactions between these infecting pathogens might also contribute to the exacerbation of AT lesions. Indeed, the simultaneous presence of *C. pneumoniae* and *H. pylori* [67] or *C. pneumoniae*, HCMV and HSV-1 [68] has been observed in AT areas of the coronary arteries.

Conclusions

Local and systemic inflammation and chronic infection with common pathogens such as *C. pneumoniae* and HCMV are strongly associated with the development of AT, a highly prevalent disease worldwide. These pathogens might also contribute to the complications of AT, including plaque instability and rupture. These associations are suggested by an increasing number of seroepidemiological data, by demonstration of the presence of DNA or proteins of these pathogens in AT plaques, and by animal models revealing the initiation or exacerbation of existing AT disease when the animals are infected with these pathogens. The effects of *in vitro* infections on the cellular components of AT plaques, vascular endothelial cells, smooth muscle cells and monocytes/macrophages, e.g. the induction of various adhesion molecules, cytokines and growth factors, indicate the mechanisms by which these pathogens function *in vivo*.

However, solid evidence for the causative role of infectious agents is missing and it seems premature to initiate anti-microbial strategies in an attempt to cure or attenuate AT diseases. In fact, a small number of clinical trials designed to test whether macrolides known to be effective against *C. pneumoniae* infections mitigate coronary artery disease have resulted in conflicting results, i.e. a reduction in clinical events in the antibiotic-treated vs the control group [69, 70], or no differences in clinical events

[71]. Additional human and animal studies to identify further pathophysiological associations among infections, inflammation and autoimmunity induced by infectious illnesses, the genetic factors of the host, and the process of development of AT are clearly needed.

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SEXUAL AND NON-SEXUAL TRANSMISSION OF HUMAN PAPILLOMAVIRUS (A SHORT REVIEW)*

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Benign tumors and lesions of the anogenital tract are caused by human papillomaviruses (HPVs). They are also major risk factors for cervical cancer. Introduction of the polymerase chain reaction (PCR) revealed that HPV infections are much more common among young asymptomatic women than it had been previously suspected.

The side-specificity of genital HPVs led to the assumption that HPVs were primarily transmitted by sexual contact. However, since HPVs have been detected in virgins, infants/children and juvenile laryngeal papillomatosis was shown to be caused by these viruses, it became acknowledged that HPVs may be transmitted by other – non-sexual – routes as well.

The evidence for sexual and different non-sexual routes of transmission of HPVs will be reviewed here.

Keyword: human papillomavirus transmission

Sexual transmission of human papillomavirus

A lot of epidemiological studies analyse genital HPV infections in a large number of cytologically normal cervical smears from women aged 15–70 years, using different techniques [1–7].

PCR detects latent, subclinical and clinical HPV manifestations, so the most reliable screening method is the combination of general-primer-mediated and type-specific PCR (GP-PCR/TS-PCR). With these tests an age-dependency was shown in

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HPV prevalence with a maximum between 20 and 24 years: statistically significant difference in HPV prevalence was found between women aged 15–34 years and women aged 35–55 [8]. Transient genital HPV infection (fluctuation, clearance) was also documented [9]. Few cases of childhood condylomata acuminata have evidence of sexual abuse [10].

Non-sexual transmission of human papillomaviruses

Vertical transmission of human papillomaviruses

Periconceptual transmission

Transmission of certain viruses to the fetus occurs most frequently during their viraemic phase by spread of infected leukocytes and macrophages. Chorionic and placental tissues may be infected by direct spread of the virus to amnionic cells that are subsequently ingested by the fetus.

The existence of a viraemic phase has not been confirmed in case of HPV infection: haematogenous transmission of the virus to the embryo/fetus and their compartments is unknown. No viral DNA was found in any chorionic villi samples collected by transabdominal sampling at the 22nd week of gestation, however, 25% of patients carried HPV in the cervix [11]. In some early first-trimester pregnancies our results confirm the HPV infection of embryonic and trophoblastic tissues even in HPV negative women (unpublished observation). The presence of HPV DNA was also demonstrated in syncytiotrophoblastic cells of first-trimester spontaneous abortion materials [12]. Earlier studies showed that Percoll-purified human sperm cells could carry HPV DNA and RNA [13, 14].

These observations indicate that the earliest HPV infection of human being might occur prior to implantation, possible by fertilization of the oocyte by an HPV carrying spermatozoon. According to our study more embryonic than trophoblastic tissues carried HPV, thus the subsequent virus-spreading might originate from the embryo.

Prenatal transmission

Several studies have reported an increase of the prevalence of genital HPV infections during pregnancy [15, 16, 17]. Hormonal changes may encourage increased transcription mediated by the glucocorticoid response elements in the non-coding

region of HPVs. Also a higher prevalence of the virus may be due to the natural transient immunosuppression, which accompanies pregnancy.

Transmission from mother to the fetus may occur in clinically apparent, subclinical and latent maternal infection. Anatomic changes in the lower uterine segment of a genital HPV infected women far gone with child and rupture or premature rupture of the membranes might lead occasionally to prenatal infections.

In third-trimester pregnancies detection of HPV DNA in amnionic fluid, cord blood and in the mucosa of neonates born by Cesarean section present evidence for a possible ascending infection [18]. A case report describes the prenatal transmission of the cutaneous HPV types 5 and 8 [19]. The delivery was by Cesarean section and amnionic fluid was taken prior to the rupture of membranes. The same variants of the virus detected in the skin of newborn were disclosed in the amnionic fluid and in the placenta.

All these variants were found in cervical scrapes – not in peripheral blood mononuclear cells – taken from the mother and in the mouth and hair of the child, examined 6 months after birth.

Perinatal transmission

It is probable that passage of the neonate through an infected birth canal or birth canal with HPV lesions explains both the source and route of HPV detection in infants [20]. However, the risk of perinatal transmission seems to be relatively low, as indicated by a prospective cohort study [21].

Women who transmit infection to their infants have significantly greater quantities of viral DNA in cervico-vaginal samples than those who do not so [22]. Several studies have revealed that HPV DNA positive specimens of infants may merely reflect passive contamination with infected maternal cells, rather than an established – transcriptionally active – infection in the infant. However, several studies have proved that HPV DNA positivity of samples on the external genitalia and/or in the buccal cavity of 24-hour-old infants, persisted in high percentage until they were 6 weeks, six month and 2 years of age, respectively. These observations suggested that replicative infections had been established usually with the same HPV genotype [23, 24, 25, 26].

Horizontal transmission of human papillomaviruses

Some viruses (e.g. HIV) may be transmitted via breast milk, but it seems unlikely that “non-bloodborn” HPVs are present in breast milk. Also there is no evidence that these viruses are associated with breast lesions.

However, a child bathing with her mother having HPV condylomata may be infected and later presents the same lesions [27]. The virus may also be transmitted via

HPV-contaminated fomites and clothing of relatives/friends [28, 29]. Numerous results also suggest that horizontal buccal infection may occur in infants and children of HPV negative mothers [30, 31].

Genital HPV infection of children

PCR studies have indicated that the prevalence of HPV type 16 DNA in the buccal cavity of children may range from 0.25–52% and ~ 17% in vulval swabs [32, 33, 34, 35, 36]. HPV16 DNA was detected in buccal swabs of 3–11 years old children in 52% using nested PCR. In some cases a transcriptionally active infection was also demonstrated [37]. Serological studies support childhood infection using HPV16 virus-like particles, as antigen. Enzyme immunoassay (EIAs) – with different HPV16 peptides, fusion proteins – have shown seroprevalence of between 4–44% among children between 0–13 years [36, 38, 39, 40–42].

In the future HPV infections – transmitted mostly by vertical routes – might be prevented by prophylactic HPV vaccines given at birth.

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IDENTIFICATION OF *SCHIZOSACCHAROMYCES POMBE* GENES THAT ENCODE PUTATIVE HOMOLOGUES OF *SACCHAROMYCES CEREVISIAE* MEDIATOR COMPLEX SUBUNITS*

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The mediator complexes transduce regulatory information from upstream regulatory elements to the transcription machinery in organisms ranging from yeasts to humans. By a genome-wide search we identified 14 ORFs and genes in the genome of the fission yeast *Schizosaccharomyces pombe* that encode putative homologues of *Saccharomyces cerevisiae* mediator subunits. The *Sch. pombe* proteins are smaller and appear to form a mediator of lower complexity, which is consistent with the hypothesized ancient origin of fission yeasts.

Keywords: fission yeast, mediator, transcription, BLAST, phylogenesis

Introduction

All eukaryotes share a highly conserved mechanism of transcription regulation. The transcription initiation of protein-encoding genes requires interactions between cis-acting promoter elements and trans-acting factors [for a review, see 1]. The promoter consists of core elements, which include the TATA box and other DNA sequences that define transcription start sites and upstream regulatory elements, which either enhance or repress transcription in a gene-specific manner. The cis-acting factors include RNA polymerase II, general transcription factors, transcription activators and coactivators.

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The general transcription factors assemble at the core promoter and include the TATA-binding protein (TBP), TFIIB, TFIIE, TFIIIF and TFIIH. Transcription activators – which bind sequence-specific regulatory elements located upstream of the core promoter – stimulate the rate of transcription in response to physiological or developmental stimuli. A third class of transcription factors, known as coactivators, facilitates the interaction between the sequence-specific activators and the general RNA polymerase II machinery. They are not gene-specific and usually mediate activation by a broad spectrum of activators. Coactivators include the TAF (TBP-associated factor) component of the general transcription factor TFIID, the mediator complex(es) (e.g. SRB/MED) that associate(s) with RNA polymerase II, TFIIA, the histone acetyltransferase complexes (e.g. SAGA), and the chromatin remodelling complexes (e.g. SWI/SNF) [for recent reviews, see 1, 2, 3].

The mediator complex (SRB/MED) discovered in *Saccharomyces cerevisiae* interacts with RNA polymerase II, forming a "holoenzyme" complex. It binds tightly to the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II and can physically interact with at least some activators. The *S. cerevisiae* mediator complex includes the Srb proteins, the Med proteins and the proteins Cse2, Gal11, Nut2, Rgr1, Rox3 and Sin4 [4, 5, 6, 7]. The components of the complex assemble into functional modules that regulate the transcription of distinct groups of genes [8, 9].

Mediator complexes have also been identified in various metazoans [reviewed in 3], indicating that the function of mediator as a coactivator for gene-specific transcription regulation is evolutionary conserved. These complexes contain homologues of several, but not all, components of the mediator of *S. cerevisiae* and also contain proteins that do not have counterparts in the budding yeast. Very recently, ten mediator proteins were also identified in the fission yeast *Schizosaccharomyces pombe* [10, 11]. Since these yeasts are almost as different from each other as from animals [12], a detailed comparison of their mediators might shed more light on the evolution of transcription regulation in eukaryotes. Here we report on the identification of homologues of the *S. cerevisiae* mediator genes in the Sanger Center database that contains the recently completed genomic *Sch. pombe* sequence (http://www.sanger.ac.uk/Projects/S_pombe). We also present an initial analysis of the data, describing some of the insight that can be obtained from the sequences.

Methods

The *S. cerevisiae* mediator sequences were used as query sequences to conduct basic BLAST searches, using the blastp (basic local alignment search technique for protein sequences) search algorithm [13], to look for proteins (translated sequences)

that are similar in *Sch. pombe* in the non-redundant database at the Sanger Center, Cambridge. Peptide sequences were compared with entries in other nonredundant proteins and nucleotide databases with the use of the National Center for Biotechnology Information Advanced BLASTP programme [14]. For pairwise comparison of sequences we used the National Center for Biotechnology Information "Blast 2 sequences" programme [15]. The phylogenetic relationships among the proteins were investigated by the PROTPARS (Protein Parsimony) programme of the PHYLIP (Phylogeny Inference Package) package, version 3.573c [16].

Results and Discussion

The *S. cerevisiae* mediator complex is composed of the Srb family of proteins (Srb2, Srb4, Srb5, Srb6 and Srb7), the Med proteins (Med1, Med2, Med4, Med6, Med8, Med9, Med10 and Med11), Cse2, Gal11, Rgr1, Rox3 and Sin4 (Table I) [4, 5, 6, 7]. Recently, two independent lines of research demonstrated that a similar complex exists in *Sch. pombe*. One of the cell separation genes, *sep15*⁺, turned out to encode a protein with high degree of sequence similarity to Med8 [11]. Independently, the purification and partial characterisation of RNA polymerase II holoenzyme from *Sch. pombe* revealed a multiprotein complex that contains homologues to Rgr1, Srb4, Med4, Med7 and Nut2 proteins, representing each of the three subgroups of mediator proteins of *S. cerevisiae* [10]. It also contained four proteins denoted Pmc2, Pmc3, Pmc5 and Pmc6 with no discernible similarity to *S. cerevisiae* subunits. Its total mass was about 400 kDa, significantly less than 1000 kDa, the mass of the *S. cerevisiae* mediator. Interestingly, no homologues were found for 17 *S. cerevisiae* mediator components. Since the composition of the isolated complex is critically dependent on the methods applied to purification, the described proteins may represent only a subset of the subunits of the *Sch. pombe* mediator complex. Different purification strategies may lead to the identification of additional components.

An alternative approach to the identification of more components can be the search of the *Sch. pombe* sequence database for genes that encode amino acid sequences similar to those of the *S. cerevisiae* subunits. With the completion of the *Sch. pombe* genome project, the whole genome has become available for homology search. In order to identify putative homologues we performed a genome-wide Blastp search in the *Sch. pombe* genome database based at the Sanger Center (<http://www.sanger.ac.uk>). As shown in Table II, matching sequences were found for all but two *S. cerevisiae* proteins. For most of them more than one *Sch. pombe* sequence producing high-scoring segment pairs was found, but for further analysis we selected

only the most similar sequence from each group. We identified all genes previously described to encode homologues of *S. cerevisiae* mediator proteins such as Med7, Med8, Nut2, Pmc1 and Srb4. We also found fourteen uncharacterized ORFs whose putative products showed various degrees of similarity to additional *S. cerevisiae* mediator proteins. In principle, these might be the genes that encode the *Sch. pombe* counterparts. We were unable to identify sequences homologous to Med1 and Med2.

Table I

Saccharomyces cerevisiae Mediator subunits¹

Subunit	Size (amino acid)	Gene
Cse2	149	CSE2, MED9
Gal11	1081	GAL11, SPT13, SDS4, RAR3
Med1	566	MED1
Med2	431	MED2
Med3	431	PGD1, HRS1
Med4	284	MED4
Med6	295	MED6, MTR32
Med7	222	MED7
Med8	223	MED8
Med11	131	MED11
Nut2	157	NUT2, MED10
Rgr1	1082	RGR1
Rox3	220	ROX3, SSN7
Sin4	974	SIN4, SSN4, TSF3
Srb2	210	SRB2
Srb4	687	SRB4
Srb5	307	SRB5
Srb6	121	SRB6
Srb7	140	SRB7
Srb8	1427	SRB8, SSN5, ARE2
Srb9	1420	SRB9, SSN2, UME2
Srb10	555	SRB10, SSN3, UME5, ARE1
Srb11	323	SRB11, SSN8, UME3

¹see references [2, 5, 6]

To verify the homologies revealed, each pair of sequences was subjected to a Blast 2 sequence comparison. This analysis did not confirm the homology between Cse2, Med3, Med11, Sin4, Srb2, Srb5, Srb9 of *S. cerevisiae* and the *Sch. pombe* sequences identified in the database search. Most probably, these proteins do not have counterparts in *Sch. pombe*. The rest of the proteins shared regions of homology with 24 to 53 % sequence identity. In certain proteins the similarity of amino acid sequences was confined to rather short regions. For example, Gal11 (1018 aa) and its putative

Sch. pombe homologue (653 aa) share only a very short (93 aa) homologous stretch (Fig. 1A). On the other hand, proteins like Med6, Med8 and Srb7 show similarity over the entire sequence (Fig. 1B, D and E). Altogether, 14 out of the 23 known *S. cerevisiae* mediator proteins seem to have homologues in *Sch. pombe*.

Table II

Schizosaccharomyces pombe Mediator subunits¹

<i>Sch. Pombe</i>			Homology with
Subunit	Size	Gene or ORF	<i>S. cerevisiae</i> subunits
Pmc4	239	SPBC1105.06	Med4
SpMed7	376	<i>med7</i>	Med7
	200	<i>sep15</i>	Med8
SpNut2	138	SPBC31F10	Nut2
Pmc1	879	<i>pmc1</i> (SPBC1A4.10C)	Rgr1
SpSrb4	545	<i>srb4</i>	Srb4
Pmc2		<i>pmc2</i> (SPAC2F7.04)	
Pmc3			
Pmc5			
Pmc6			

¹see references [10, 11]

Remarkably, 12 out of the 14 *Sch. pombe* proteins are smaller than their *S. cerevisiae* counterparts, which fits in fairly well with the earlier finding that the *Sch. pombe* mediator is smaller [10]. The smaller size of the *Sch. pombe* proteins might be attributed to the hypothesized ancient origin of the fission yeasts [12]. One can speculate that these proteins contain little more than the domains necessary for the basic activity, whereas the *S. cerevisiae* proteins have acquired longer sequence extensions required for specific tuning of the activity. With the exception of Med6, all *S. cerevisiae* proteins have these non-homologous extensions at their ends (examples are shown in Fig. 1). In Med6 the region of homology is split up in four parts by a shorter and two longer unique stretches that are absent in the *Sch. pombe* sequence (Fig. 1B). Med7 represents an exception to the rule that the *S. cerevisiae* proteins are longer. Its counterpart in *Sch. pombe* has a unique internal block and a unique terminal stretch that make the whole amino acid sequence longer than that of the *S. cerevisiae* Med7. It seems likely that the homologous regions separated by the internal block in *Sch. pombe* correspond to two specific protein domains.

Table III

Mediator homologs identified in the Sanger database of the Sch. pombe genome project

<i>Saccharomyces cerevisiae</i>		<i>Schizosaccharomyces pombe</i> homologs in Sanger database ¹			Sequence homology <i>Sch. pombe</i> / <i>S. cerevisiae</i>	
Subunit	Size (amino acid)	Gene or ORF	BLASTP value ²	Size (amino acid)	identity	positives
Cse2	149	SPAC10F6.09C	0.66	1234	no significant similarity	
Gal11	1081	SPBP35G2.15	9.8e-06	653	28/93 (30%)	46/93 (49%)
Med1	566	no hit				
Med2	431	no hit				
Med3	431	SPBC800.06	0.9992	305	no significant similarity	
Med4	284	SPBC1105.06	1.3e-06	239	34/125 (27%)	64/125 (51%)
Med6	295	SPAC1002.15c	1.7e-09	216	63/214 (29%)	99/214 (45%)
Med7	222	SPBC14F5.08	1.2e-18	376	82/314 (26%)	127/314 (40%)
Med8	223	<i>sep15</i>	2.1e-11	200	58/191 (30%)	100/191 (51%)
Med11	131	SPAC6G9.06C	0.0075	1208	no significant similarity	
Nut2	157	SPBC31F10	2.7e-13	138	40/150 (26%)	76/150 (50%)
Rgr1	1082	SPBP23A10.01C	1.2e-13	879	96/357 (26%)	158/357 (43%)
Rox3	220	SPCC1450.05C	0.0042	138	24/60 (40%)	37/60 (61%)
Sin4	974	SPBC1347.01C	0.9992	935	no significant similarity	
Srb2	210	<i>Lys1</i>	0.98	1415	no significant similarity	
Srb4	687	SPBC31F10.04C	1.4e-08	545	47/137 (34%)	78/137 (56%)
Srb5	307	SPAC1327.01C	0.11	977	No significant similarity	
Srb6	121	SPAC29A4.07	0.061	136	25/100 (25%)	44/100 (44%)
Srb7	140	SPBC1604.10	0.019	138	41/129 (31%)	67/129 (51%)
Srb8	1427	SPAC688.08	6.8e-09	1233	52/214 (24%)	91/214 (42%)
Srb9	1420	SPBC30D10.15	0.83	516	No significant similarity	
Srb10	555	SPAC23H4.17C	2.6e-92	352	160/299 (53%)	215/299 (71%)
Srb11	323	SPBC12D12.06	4.4e-17	228	63/163 (38%)	99/163 (60%)

¹<http://www.sanger.ac.uk>²smallest sum probability

A

Gall1Sp: 349 EMLNIPVPKQLFDQIPNLPNPKIWRDVLGLGQSQRLPPEQLKLGMLYRXXXXXXXXX 408
 +M P+PKQL +IPN+PPN+ W+ V L Q + L P+ ++ +Y+
 Gall1Sc: 172 QMKVAPIPKQLLRIPNIPPNTWQQVTLAQQLLTPQDMEAAKEVYK-----IHQQ 225
 Gall1Sp: 409 XXXXNKIQNARMNSQNAPNTNKLGNPQPDNTGN 441
 ++Q + +Q N N G PQ N N
 Gall1Sc: 226 LLFKARLQQQAQAQAQANNNNGLPQNGNINN 258

B

Med6Sp: 11 LTSIQWRMPPEWVQSMGGLRTENVLEYFSQSPFYSHKSNNEMLMQSQF----- 58
 L +QW+ PEW+Q G LRTEENVL+YF++SPF+ SNN+++KMQ QF
 Med6Sc: 6 LDELQWKSPEWIVQFG-LRTEENVLDYFAESPFDDKTSNNQVIKMQRQFSQLNDPNAAVNM 64
 Med6Sp: 59 --NALDLGD-----LNSQLKRLTGIFVVIHERPPF 87
 N + L D L +L +L G ++V+ R P
 Med6Sc: 65 TQNIMTLDPGKNGNLEEFAYVDPARRQILFKYPMYQLEELMKLDGTEYVLSVREPD 124
 Med6Sp: 88 LWVIQKQNRNLNEN-----EVKPLTVYFCNENIYMAPNAYTLATRLNATYCFQKA 139
 WVI+KQ R N + E+ PL Y++ NIY +P + ++ +R+++ +Y
 Med6Sc: 125 FWVIRKQRRNTNSGVSAKGPETIPLQDYIIGANIYQSPTIFKIVQSRLMSTSYHLNST 184
 Med6Sp: 140 LTKIEKFPQYNPQEGYTPKLSNDNLEVDHSNTN 173
 L + ++ P +G Y K+ D + TN
 Med6Sc: 185 LESLYDLIEFQPSQGVHY-KVPTDTSTTATAATN 217

C

Med7Sp: 1 MEPNEGVLFSAPPPPPYYKLFTRENIEKVISNMEKEAKHEDDA--NTLQPKTEEEIES 58
 M + G ++ S +PPPPY K FT+ N+EK+ EK+A N +EEEI
 Med7Sc: 1 MSNDPGNEVSSLYPPPPYVKFFTQSNLEKLPKYKEKKAASAKQTAPNNSNGGSEEBITC 60
 Med7Sp: 59 LAKLFKKPSCLTSGTYQMFGDTWRLEDAIPSLKEFGIPELYKDIKDGEDEIEVVEYDPKS 118
 P + Y+ FG W++ + +P L+ G+ +LYK
 Med7Sc: 61 ALDYLIPPMPKNNQYRAFGSIQVKDQLPDLESMGLTQLYK----- 102
 Med7Sp: 119 NAIVGTSFTRVHDYDKNSPIENKILEDDQHTAMKEKDNECDKTMKDVEEKTEPSLKKEE 178
 K +NES ++E
 Med7Sc: 103 -----KSTENESTNYQYKIQE----- 118
 Med7Sp: 179 EDIQMKEPLDSQDTGAVSASSVNEGFRADQKSKDGETSLLIKIPRAYELRFLSRSLMLN 238
 LR L +SL+LN
 Med7Sc: 119 -----LRKLLKSLLLN 129
 Med7Sp: 239 FLELLGIMAKAPEQFSPKVENIRVLLNLHLLINDYRPHQSRESLIMLLEKQLKHEESQV 298
 +LEL+G+++ P+ + KVENIR +L+N+HHL+N+YRPHQSRESLIMLLE+QL+++ ++
 Med7Sc: 130 YLELIGVLSINPDMYERKVENIRITLVNIHLLNEYRPHQSRESLIMLLEEQLEYKRGEI 189
 Med7Sp: 299 ELLRTHNRQMTETL 312
 + +Q+ + L
 Med7Sc: 190 REIEQVCKQVHDKL 203

Fig. 1. Sequence comparison of *S. cerevisiae* mediator subunits and their putative *S. pombe* homologues. Comparison of Gall1Sp with Gall1Sc (A), Med6Sp with Med6Sc (B), Med7Sp with Med7Sc (C), Med8Sp with Med8Sc (D), Srb7Sp with Srb7Sc (E), Srb10Sp with Srb10Sc (F), and Srb11Sp with Srb11Sc (G). Symbols of amino acids are A: alanine, C: cysteine, D: aspartic acid, E: glutamic acid, F: phenylalanine, G: glycine, H: histidine, I: Isoleucine, L: leucine, K: lysine, M: methionine, N: asparagine, P: proline, Q: glutamine, R: arginine, S: serine, T: threonine, W: tryptophane, Y: tyrosine, V: valine

D

Med8Sp: 11 ESLEAIRHRIAQIVQSLTHFLAILHQSESLSPWPTIHKNFNILLSQIHSLSNLAASHST 70
 ++L+A+R R+AQ+ SL + ++E L W T+ N+ LSQ+ S+++ L T
 Med8Sc: 31 QALDAVRMLAQLTHSLRRIRDEMSKAE-LPQWYTLQSQLNVTLSQLVSVTSTLQHFQET 89
 Med8Sp: 71 LQTTISIYPSLEFPVKEQEPLLTLLRTKALPEVEWEANTLQEYEASISSQPKKKEANDA 130
 L +T +YP +FP E L+TTLLR K +PEV+EW ++E ++ K +E
 Med8Sc: 90 LDSTVVYPLPKFPPTSHESLVTLLRKKNIPEVDEW-MKYVRETSGVTTALLKDEEIEKL 148
 Med8Sp: 131 YQKD-QLWDQARIIFMEERENYSWFDFVTRRQSESEGEFVSQRQLEIDRATEEQNANQMLT 189
 Q+D ++ + AR F R Y DF + +ES E + + + ++ N +
 Med8Sc: 149 LQQDREITNWARTTF---RNEYGKHDF--KNEESLSEEHASLLVRDSKPSKPFNVND---- 199
 Med8Sp: 190 DILSFMKSGKR 200
 D+L F +G++
 Med8Sc: 200 DVLKFTFTGEK 210

E

Srb7Sp: 1 MACRCTQLQDTIDEVATQFYSSIIHLYSSHDFVPLPGQE-KVSDSKVNPISAEELQFAQR 59
 M R TQLQ +D++ QF ++++Y+ +H F L E ++SD + EE
 Srb7Sc: 1 MTDRLTQLQICLDQMTEQFCATLNYIDKNHGFERLTVPNEPQMSDKHATVVPPEEFSNTID 60
 Srb7Sp: 60 DLAKDLVTKFMQIDTLINQLPGISTAPKHQLEKIKKLQNSIEEKQLERKSLESENEDLKL 119
 +L+ D++ K QI+ LI+ LPG+ + + QL KI LQ + E + E+ + E L
 Srb7Sc: 61 ELSTDIILKTRQINKLIDSLPGVDVSAEEQLRKIDMLQKKLVEVEDEKIEAIKKKEKLLR 120
 Srb7Sp: 120 QLAKRIETF 128
 + IE F
 Srb7Sc: 121 HVDSLIEDF 129

F

Srb10Sp: 33 FAIKKFKAESKQVSSNAQQTGVSQSAIREMMLCREIQHENIVSLVQVLLKDGITISMVFY 92
 +AIKKFK E V TG+SQSA REM LCRE+ ++++ +LV++ L+ + MV+EY
 Srb10Sc: 166 YAIKKFKTEKDGVEQ-LHYTGISQSAIREMALCRELHNKHLTTLVEIFLERKCVHMYEY 224
 Srb10Sp: 93 AEHDLLQIIHFHSRSRTRQIPPSILKSILWQIINGVAYLHENWIMHRDLKPANIMITATG 152
 AEHDLLQIIHFHS R IPP +++SI+WQ+++GV+YLH+NW++HRDLKPANIM+T G
 Srb10Sc: 225 AEHDLLQIIHFHSHPEKRMIPPRMVRSIMWQLLDGVSYLHQNWWLHRDLKPANIMVTIDG 284
 Srb10Sp: 153 KVKIGDLGLRLIRDPILPFYSSDRVVVTIWIYRAPELLLAGHDYTPAIDVWAIGCIYEM 212
 VKIGDLGL R + + Y+ D+VVVTIWIYRAPELLGA YTPA+D+W++GCI+ E+
 Srb10Sc: 285 CVKIGDLGLARKFHNMLQTLTYTGDKVVVTIWIYRAPELLGARHYTPAVDLWSVGCI PAEL 344
 Srb10Sp: 213 LALSPLFKGDEIKMEDKKVVPFQSTQMLRIMELLGTPTEERWPGLKNYPEYYQLSSFEVR 272
 + L P+FKG+E K++ KK VPFQ Q+ RI+E+LGTP ++ WP L+ YPEY Q++ F +
 Srb10Sc: 345 IGLQPIFKGEEAKLDSKKTVPFQVNLQRLILEVLGTPDQKIWPYLEKYPEYDQITKFP-K 403
 Srb10Sp: 273 YWNNLLPQWYQTVKNRDPQGLDLLMKMLQYDPKSRITAKALEHVFFTSCLKWITRYVF 331
 Y +N L WY + RD L LL +L YDP RI A ALEH +FT + + VF
 Srb10Sc: 404 YRDN-LATWYHSAGGRDKHALSLLYHLLNYDPIKRIDAFNALEHKYFTESDIPVSENVF 461

Fig. 1. (continued)

G

Srb11/SRB11
 Srb11Sp: 33 IYQWKVVQTFGDRRLRLRQVLATAIVLLRRYMLKKNEEKGFSLVATCIYLSCKVEEC 92
 IY + ++ G RL +RQ LATA + L R+++K + + +L LV TC+YL+CKVEEC
 Srb11Sc: 76 IYCYFLIMKLGRRLNIRQYALATAHIYLSRFLIKASVRE-INLYMLVTTTCVYLACKVEEC 134
 Srb11Sp: 93 PVHIRTICNEANDLWSLKVKLXXXXXXXXXXXXXVLDAPFLIVHHPYTSLEQAFH----- 147
 P +IRT+ +EA LW + L+++LIVHHPY SL+Q
 Srb11Sc: 135 PQYIRTLVSEARTLWPEFIPDPPTKVTEFEFYLLLEELESYLIVHHPYQSLKQIVQVLKQP 194
 Srb11Sp: 148 --DGIINQKQLEFAWSIVNDSYASSLCLMAHPHQLAYAALLIS 188
 ++ L+ WS++NSY + + L+ PH +A A L I+
 Srb11Sc: 195 PFQITLSSDDLQNCWSLINDSYINDVHLLYPPIIIVACLFIT 237

Fig. 1 (continued)

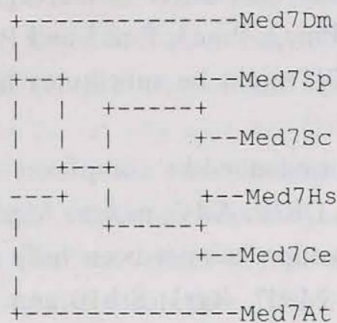


Fig. 2. Maximum parsimony tree for the *S. cerevisiae* Med7 protein (Med7Sc) and its homologues in *Arabidopsis thaliana* (Med7At), *Cenorhabditis elegans* (Med7Ce), *Drosophila melanogaster* (Med7Dm), *Homo sapiens* (Med7Hs) and *Sch. pombe* (Med7Sp)

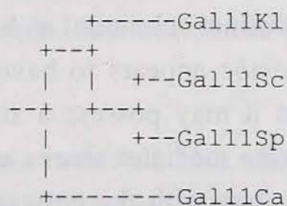


Fig. 3. A maximum parsimony tree inferred from the sequences of the *S. cerevisiae* Gal11 protein (Gal11Sc) and its homologues in *Candida albicans* (Gal11Ca), *Kuyveromyces lactis* (Gal11Kl) and *Sch. pombe* (Gal11Sp)

Recent studies indicate that mediator complexes are organized in a modular fashion [reviewed in 3]. In *S. cerevisiae* the core mediator subunits form two submodules, Cse2-Med1-Med2-Med4-Med7-Med8-Nut2-Rgr1-Srb7 and Med6-Rox3-Srb2-Srb4-Srb5-Srb6 [17], that interact with the modules composed of Gal11-Med3-Sin4 and Srb8-Srb9-Srb10-Srb11 [18]. Combined biochemical and genetic studies demonstrated that specific functions can be attributed to each subcomplex [19]. For example, *SRB10* and *SRB11* encode a kinase-cyclin pair involved in the phosphorylation of CTD in a cell cycle-dependent manner [20, 21]. We found putative *Sch. pombe* homologues of components of all these modules, indicating that the *Sch. pombe* mediator performs functions similar to those of the *S. cerevisiae* mediator. However, none of the modules are completely represented in *Sch. pombe*, which suggests that the two yeasts may also differ in the organisation and activity of their mediators. The Pmc proteins (Pmc2, Pmc3, Pmc5 and Pmc6), for which no homology was found in *S. cerevisiae* [10], might be substitutes for the missing homologues in these modules.

Numerous mammalian mediator-like complexes have been identified recently, including SMCC/TRAP, NAT, DRIP, ARC, murine Mediator and CRSP [for a review, see 3]. Their molecular composition has not been fully defined but they all appear to contain homologues of Med6, Med7, Rgr1, Srb10 and Srb11. *Cenorhabditis elegans* also has genes that code for homologues of Med6, Med7, Rgr1, Srb7 [22]. We found the counterparts for these genes in *Sch. pombe*, too, which indicates that these proteins may constitute the key mediator components that are evolutionarily conserved from yeasts to mammals. Remarkably, none of the mammalian and *C. elegans* complexes described to date contain readily apparent homologues of yeast Srb2, Srb4, Srb5 or Srb6. These proteins constitute a distinct submodul in *S. cerevisiae* and are critical for activator interactions [23]. *Sch. pombe* appears to have two members of this group, Srb2 and Srb5, demonstrating that it may possess a similar submodul. This finding further indicates that the *S. cerevisiae* mediator shares more features in common with the *Sch. pombe* mediator complex than with the corresponding metazoan complexes. We hypothesized that a closer relationship should also be reflected in a higher degree of sequence similarity between mediator proteins of these yeasts. To test this idea, we tried to find homologues for them in as many species as possible by searching various sequence data bases. Srb10 showed high degree of homology to a wide spectrum of cyclin dependent protein kinases of a great number of species. Many proteins with highly similar sequences were also found for Srb11, which were mostly cyclins. However, the limited information available about the exact functions of many of these kinases and cyclins did not allow the identification of the real homologues. Therefore we did not use them for multiple sequence comparison. Instead, we choose Med7, for

which single highly similar homologues were found in three Metazoans and one plant species. The most parsimonious tree shown in Fig. 2. indicates a close relationship between the yeast proteins.

A search of the National Center for Biotechnology Information data base revealed Gal11 homologues only in yeasts. In addition to *S. cerevisiae* and *Sch. pombe*, genes encoding Gal11-like proteins were also identified in *Candida albicans* and *Kluyveromyces lactis*. Previously, it was suggested that Gal11 is absent in *Sch. pombe* because the RNA polymerase II holoenzyme does not contain a protein with molecular mass similar to that of *S. cerevisiae* Gal11 [10]. Its absence seemed to correlate with the inability of *Sch. pombe* to grow on galactose. In *S. cerevisiae*, the Gal11 protein is needed to support Gal4-dependent induction of genes involved in galactose metabolism [16]. Interestingly, we could find an ORF encoding an amino acid sequence that shares high degree of sequence similarity with Gal11 over a 93-amino acid region (Fig. 1A). This hypothetical protein is significantly shorter (653 aa) than Gal11 (1018 aa), which might account for the failure of its identification in SDS-PAGE analysis of the purified polymerase II holoenzyme [10]. In spite of the considerable size difference, it appears to be more closely related to Gal11 than their homologues in the other yeast species.

In this analysis we identified numerous *Sch. pombe* genes that code for putative homologues of *S. cerevisiae* mediator subunits. The conclusion one may draw from these results is that the components of the putative *Sch. pombe* mediator complex show a higher degree of similarity with those of *S. cerevisiae* mediator than with the components of the metazoan mediators. However, the revealed sequence homologies do not necessarily coincide with functional homologies, and certain similarities may be only accidental. Future experimental studies will test which of the identified genes code for *Sch. pombe* mediator subunits and how these proteins work together to regulate transcription.

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SEARCHING FOR NEW-TYPE ANTIFUNGAL DRUGS

(AN OUTLINE FOR POSSIBLE NEW STRATEGIES)*

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New approaches for treatment of invasive fungal infections are necessary to cope with emerging resistant fungal pathogens of humans. In this paper, three different strategies are presented and evaluated to find new-type antifungal drugs and their targets. While experimental data obtained with potent chitinase inhibitors, e.g. allosamidin, and small-size antifungal proteins of fungal origin are encouraging more efforts are needed to verify and exploit the possible involvement of intracellular thiols, e.g. glutathione, and their metabolic enzymes in the pathogenesis of mycoses caused by dimorphic fungi. Chitinase inhibitors seem to hinder the cell separation of yeasts and the fragmentation of filamentous fungi quite effectively and, hence, they may be implicated in future therapies of systemic mycoses. In addition, small-size antifungal proteins possessing a broad inhibition spectrum may also provide us with promising new agents for the treatment of different kinds of (e.g. cutaneous) fungal infections.

Keyword: antifungal chemotherapy

Introduction

The incidence of invasive fungal infections rises steadily in humans starting from the 70s [1]. Patients receiving immunosuppressive therapies, using broad-

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spectrum antibacterial medicines for prolonged periods of time or suffering of immunosuppressive viral infections such as AIDS are especially predisposed to different kinds of mycoses. The number of nosocomial infections is also increasing sharply due to, for example, the common use of indwelling intravenous devices [1]. Recently used antifungal agents include ergosterol biosynthesis inhibitors, polyenes and 5-fluorocytosine [1, 2] but new targets and drug delivery systems are considered for antifungal therapy [3]. The development of new, safer and more effective antifungal drugs is stimulated by the undesirable side-effects of currently available medicines as well as the emergence of new pathogenic species possessing intrinsic primary resistance and common pathogens rapidly developing secondary resistance to these agents [2, 4]. It is worth noting the threatening emergence of naturally resistant or even polyresistant non-albicans *Candida* species, e.g. *Candida krusei* in cancer patients [4] and during antifungal therapy [5]. In this paper, we outline the efforts we made in the last couple of years to find novel antifungal agents and define new targets for future antifungal drug research.

Chitinolytic enzymes

Compounds active against fungal cell wall biosynthetic enzymes including chitin synthetases and glucan synthetases are regarded as potential and promising targets for antifungal agents including nikkomycins and echinocandins [1]. Surprisingly, the endogenous cell wall degrading enzymes of these organisms, to which versatile and important nutritional, morphological and physiological functions are attributed [6, 7], attracted much less attention until now. For example, all chitin-containing fungi studied thus far have been shown to produce chitinases that take part in swelling and germination of spores, branching and apical extension of growing hyphae, tailoring chitin microfibrils in rigidifying wall, cell separation of yeasts as well as age-related fragmentation and autolysis of hyphae [6–10]. Obviously, these important chitinolytic enzymes could be suitable targets for antifungal drug design in addition to cell wall biosynthetic enzymes.

We have already had some encouraging *in vivo* enzyme inhibition data in our hands concerning fungal chitinases. For example, the pseudotrisaccharide chitinase inhibitor allosamidin (Fig. 1) hindered effectively the cell separation of the yeasts *Saccharomyces cerevisiae* [11, 12] and *Candida albicans* [6], and the germination of some fungal spores was also found to be allosamidin-sensitive [6, 7, 13].

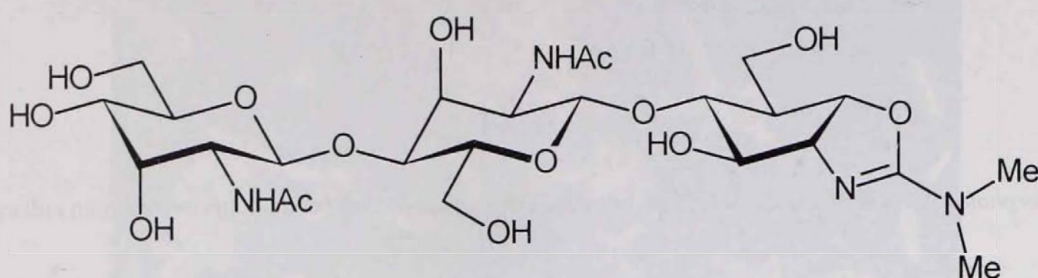


Fig. 1. Pseudotrisaccharide structure of allosamidin, one of the most potent known inhibitors of chitinases

On the other hand, allosamidin did not influence the growth and antibiotic production of the β -lactam producer filamentous fungi *Acremonium chrysogenum* [14] and *Penicillium chrysogenum* [15] (Table I). Importantly, this pseudotrisaccharide hindered significantly the idiophasic breakage of *A. chrysogenum* hyphae [14] as well as the fragmentation and autolysis observable in carbon-depleted *P. chrysogenum* cultures [15]. Allosamidin also inhibited the outgrowth of surviving hyphal fragments in autolysing *P. chrysogenum* cultures when carbon starvation was abolished by the addition of an extra dose of glucose (Figs 2 and 3) [15]. To the best of our knowledge, this was the first presentation of a possible antifungal effect of allosamidin on a filamentous growth form.

Most recent experimental data summarized in Table I clearly indicated that the antifungal effect of allosamidin on autolysing *P. chrysogenum* mycelia was fungistatic rather than fungicidal [16]. To sum it up, allosamidin and its semisynthetic derivatives [10, 17] might be effective against filamentous plant, insect or even human pathogenic fungi when cells possessing yeast or yeast-like morphology are thought to play an important role in the invasion of host tissues [18].

Possible glutathione-dependent cellular signalling pathways leading to yeast $\leftrightarrow$ mycelium transitions

In dimorphic fungi including plant, insect and human pathogenic species, morphological transitions can be provoked by a large constellation of environmental parameters [18, 19]. Usually, the pathogenicity of these micro-organisms is limited to only one of the morphological forms, and yeast $\leftrightarrow$ mycelium morphological switches

are likely to play a crucially important role in the pathogenesis of fungal infections [18, 19].

Table I

*Morphological and physiological effects of allosamidin on autolysing *Penicillium chrysogenum* cultures [15, 16]*

Characteristic	Effect of allosamidin ¹
Autolytic loss of biomass	decreases
Number of hyphal elements	increases
Chitin content of the walls	increases
Release of chitinolytic enzymes	does not influence
Vitality estimated by viable cell counts	does not influence
Vitality tested by transferring allosamidin-treated mycelia into allosamidin-free culture media (gains in cell dry weights and outgrowth frequencies)	does not influence
Intracellular glutathione, glutathione disulphide levels and glutathione/glutathione disulphide redox balances	does not influence
Outgrowth after glucose supplementation	inhibits
Glucose utilisation rates in allosamidin-treated cultures after glucose re-addition	reduces
Acidification rates in allosamidin-treated cultures after glucose re-addition	reduces
Utilisation of intracellular glutathione reserves after carbon supplementation	reduces

¹ Changes were recorded in the presence of 9.6 μM allosamidin between 115 and 167 h incubation times (autolytic and cryptic growth phases). Allosamidin was added at 35 h in the deceleration phase of growth. An extra 51 mM glucose was added to selected cultures at 115 h of incubation.

Remarkably, changes in the intracellular glutathione concentrations and glutathione/glutathione disulphide redox balances are also provoked by versatile environmental factors [20–22]. It is reasonable that the actual redox state of the cellular thiol-disulphide buffer systems will modulate the intracellular protein thiol levels and, hence, the biological activity of specific proteins involved in the regulation of dimorphic switches [23–25].

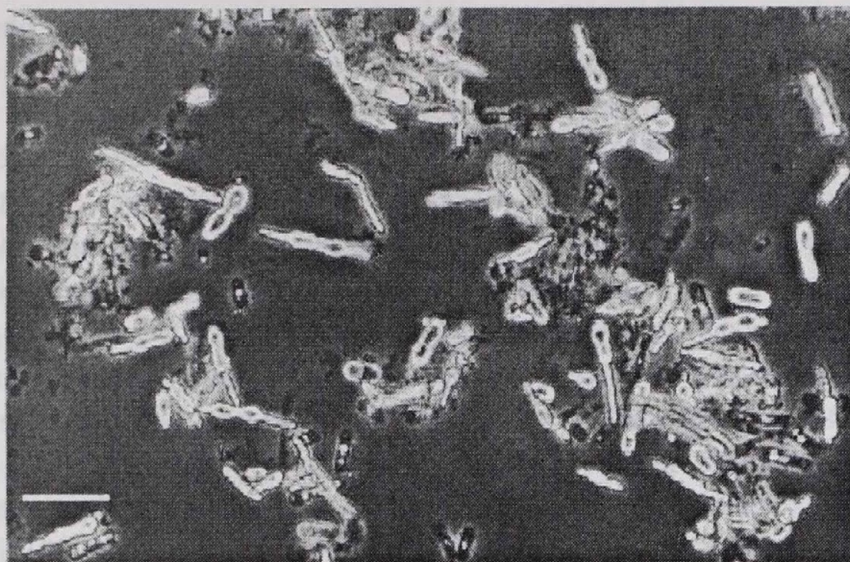


Fig. 2. Outgrowing hyphae in *P. chrysogenum* cultures (160 h) supplied with 70 mM extra glucose at 115 h fermentation time. Bar = 40 μ m. Reproduced with permission from [15]. © University of Potsdam

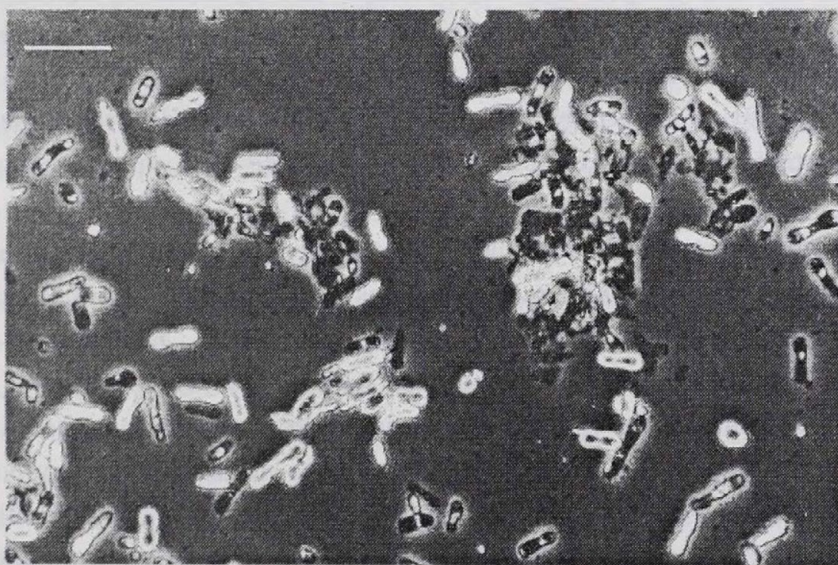


Fig. 3. A *P. chrysogenum* culture (160 h) supplemented with 9.6 μ M allosamidin (35 h fermentation time) and 70 mM glucose (115 h fermentation time). Bar = 40 μ m. Reproduced with permission from [15].

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More recently, diamide, a widely used chemical to disturb the glutathione/glutathione disulphide redox status of the cells by oxidizing glutathione to glutathione disulphide instantaneously and stoichiometrically [26, 27], was shown to have pleiotropic effects on the gene expression profile of *S. cerevisiae* [28]. In addition to protein unfolding and the generation of oxidative stress, diamide was likely to affect disadvantageously the secretion and assemble of cell wall constituents due to improper disulphide bond formation in the endoplasmic reticulum [28]. Changes in the thiol redox status of the cells may therefore have a direct effect on the cell wall structure, which should, of course, result in changes in the observed cell morphology as well. Obviously, any successful attempt to influence or disturb dimorphic transitions *via* affecting the glutathione metabolism of fungi would attract a great deal of interest from people involved in the development of new antifungal drugs.

The glutathione metabolisms of *Aureobasidium pullulans* cells possessing either yeast or mycelial morphology has been studied and compared by us [29]. This micro-organism is a weak plant pathogenic fungus normally inhabiting the surface of living or senescing leaves exploiting the more readily utilisable nutrients [18, 30]. Importantly, the cell morphology observed in *A. pullulans* cultures can be influenced by changing solely the inoculum size [31, 32], which makes this micro-organism an ideal model for the investigation of the physiological background of dimorphic transitions.

In accordance with previous experimental data obtained in *Candida albicans* cultures [23–25], significantly higher specific glutathione concentrations were found in *A. pullulans* yeast cells than in mycelia {51–55 mmol (kg protein⁻¹) vs. 36–41 mmol (kg protein⁻¹)} [29]. Surprisingly, there was no significant difference between the glutathione/glutathione disulphide redox status of the cells with either yeast or mycelial morphology.

These experimental data challenge the hypothesis that changes in cellular glutathione/glutathione disulphide redox balances would be involved in the initiation of dimorphic morphological transitions. Although some morphology-dependent differences were recorded in the specific activities of some glutathione metabolic enzymes (e.g. glutathione reductase, γ -glutamyltranspeptidase) they were not satisfactory to explain the observed alterations in the intracellular glutathione levels. Moreover, in some cases the specific activities differed more significantly in cells sharing the same morphology but separated from pure or mixed morphology cultures than those found in yeast cells and hyphae of a mixed morphology culture [29].

According to these data, the hypothesized effect of intracellular glutathione levels and glutathione metabolic enzymes on the observed cell morphology needs further verification. Taking into consideration of other components of the intracellular thiol pool including cysteine/cystine [33, 34] would give us a more reliable overview

on the role that reduced thiols and thiol buffer systems may play in the initiation and signal transduction events of dimorphic transitions.

Small-size antifungal proteins secreted by fungi

Small molecular mass (about $M_r=6000$) cysteine-rich antifungal peptides have been shown to be secreted by *Aspergillus giganteus* [35–38], *P. chrysogenum* [39], *Aspergillus niger* [40] and *Penicillium nalgiovense* [41]. It is remarkable that the *A. niger* antifungal protein shares some common sequential and structural motifs with antifungal proteins of plant origin [40], including *Arabidopsis thaliana* [42] and *Sinapis alba* [43]. Although the mechanism of action of fungal proteins has remained to be elucidated the permeabilisation of fungal cell membranes by the analogous plant proteins, defensins and thionins are well-characterized [44, 45]. The cytotoxicity of fungal proteins may also involve interaction with the phospholipid components of the cell membranes [38, 41].

As far as the inhibition spectrum is concerned, the antifungal protein of *A. giganteus* inhibited the growth of *Trichoderma koningii* (MIC=6 μM), *Fusarium oxysporum* (MIC=7–25 μM), *Penicillium purpurogenum* (MIC=10 μM) and *Trichoderma harzianum* (MIC=127 μM) [38]. This protein did not influence the growth of yeasts including *C. albicans*, *S. cerevisiae*, *Saccharomyces exiguus*, *Rhodotorula mucilaginosa* and *Pichia membranaefaciens* and was ineffective against *A. niger*, *P. chrysogenum* and *A. giganteus*, i.e. the producing micro-organism itself. The antifungal protein of *P. chrysogenum* was effective against *A. niger* [39] and *P. nalgiovense* exhibited antagonistic activity against a range of filamentous fungi [40]. It is worth noting that the antifungal protein of *A. niger* (Fig. 4) was active against the yeasts *S. cerevisiae* (MIC=8 μM) and *C. albicans* (MIC=8–15 μM) in addition to numerous filamentous species including *Aspergillus flavus*, *A. fumigatus*, *F. oxysporum*, *Fusarium solani* and *Trichosporon beigelii* (MIC=4–15 μM). Interestingly, no literature data on the possible cytotoxic effects of these antifungal proteins on human or animal cell lines and model organisms have been published thus far but the application of the *P. nalgiovense* protein for biopreservation purposes is being considered [41].

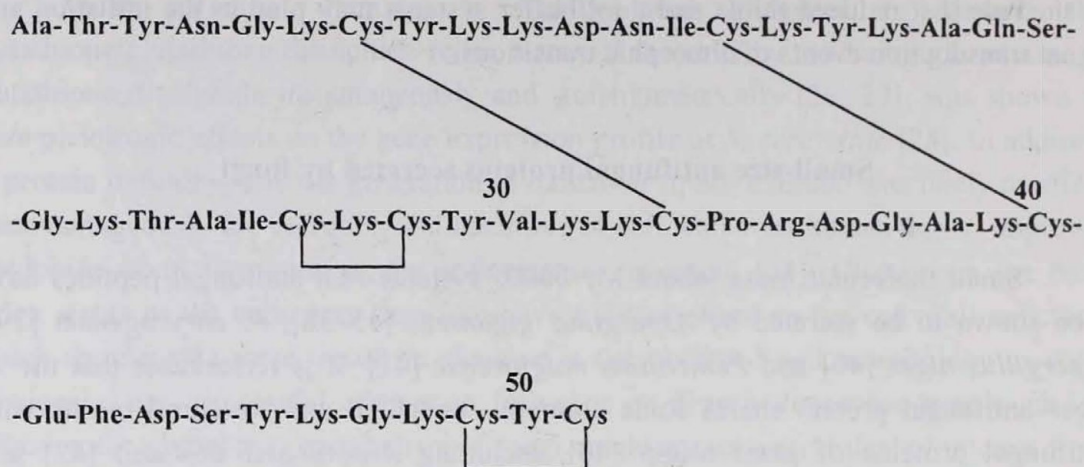


Fig. 4. Chemical structure of the small-size antifungal protein of *Aspergillus giganteus* [35]. Solid lines indicate the positions of disulphide bridges

Regarding that the inhibitory activity and spectrum of antifungal proteins are versatile even in case of closely related species it seems to be definitely worth isolating and testing the antifungal activity of further small-size proteins of this kind. A joint screening program is now in progress in our laboratories.

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MOTHER-TO-CHILD TRANSMISSION OF HIV-1: THE ROLE OF HIV-1 VARIABILITY AND THE PLACENTAL BARRIER*

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The acquired immunodeficiency syndrome (AIDS), which is caused by the human immunodeficiency virus (HIV), was first described in the United States of America in 1981 [1]. The worldwide spread of HIV has soon been recognized and AIDS has become one of the most alarming infectious diseases of our days. Its impact has been tremendous, high morbidity and mortality has caused a reversal of socioeconomic gains previously recorded in several developing countries, especially those in Sub-Saharan Africa [2].

Epidemiological data about the HIV and AIDS pandemic are updated by the Joint United Nation Programme on HIV/AIDS, UNAIDS (<http://www.unaids.org>). Their latest report from December 2000 states that in year 2000 approximately 5.3 million people have become newly infected with HIV, of which 2.2 were women and 600 000 children younger than 15 years of age. The estimated number of people living with HIV/AIDS globally is 36.1 million, of which 16.4 million are women and 1.4 million are children younger than 15 years of age. Approximately 25.3 million (70%) of these HIV infected people live in Sub-Saharan Africa, 5.8 million in South- and South-East Asia (15%), and 1.4 million in Latin-America (5%). During year 2000, 3 million people died of AIDS (1.3 million women and 500 000 children younger than 15 years of age). This means that an estimated total of 21.8 million persons have died of AIDS since the beginning of the epidemic, including 4.3 million children younger than 15 years of age.

Keywords: HIV-1, HIV-1 variability, vertical transmission, placental barrier

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The course of HIV-1 infection

HIV-1 infection in adults can be divided into three clinical stages: 1) an acute phase with pronounced viremia and with or without clinical symptoms of primary HIV-1 infection, 2) a chronic phase with minimal, but detectable, clinical and immunological abnormalities in the presence of less viremia and 3) a late stage with profound immunodeficiency which is often accompanied by opportunistic infections and/or neoplasias [3].

Disease progression is mainly monitored by registration of clinical symptoms and measurements of plasma HIV-1 RNA level and CD4+ and CD8+ T-lymphocyte counts. A decrease in CD4+ lymphocyte counts and CD4+/CD8+ lymphocyte ratios indicates progressive disease. The immunological deterioration is an important marker for disease progression, but all available data indicate that the plasma HIV-1 RNA level is the best prognostic marker [4] and the best instrument for monitoring antiretroviral therapy [5, 6].

The rate of CD4 cell decline is closely linked to the plasma HIV-1 levels, but the underlying reasons for CD4 cell destructions are incompletely understood (for review see [7]). However, it is clear that interdependent host, viral and environmental factors are of importance. Here we will focus on HIV-1 infection in the pregnant woman and in children.

HIV-1 infection during pregnancy

In normal pregnancy, the humoral response is normal but the cellular immunity has been shown to be impaired [8–10]. Thus, the number of CD4 cells and CD4+/CD8+ ratios are decreased, especially in early pregnancy [11–13]. This is thought to be favourable for accepting the fetus, which carries foreign paternal antigens.

In women with intercurrent disease such as diabetes, glomerulonephritis and cardiopathy, pregnancy can accelerate the course of the disease. Likewise, some infectious diseases such as tuberculosis, malaria and polio have been reported to carry increased morbidity and mortality in pregnant women in comparison to non-pregnant women [9]. This question has been carefully considered also in HIV-1 infected pregnant women.

Early case reports of HIV infected women with opportunistic infections during pregnancy suggested an acceleration of disease progression [14, 15]. However, these women had an advanced HIV disease. These findings have not been confirmed in later studies on mainly asymptomatic women [16–19]. Nevertheless, immunological markers are subject to large variability during pregnancy in both uninfected and HIV-1

infected women. It should also be mentioned that development of AIDS is more problematic in a pregnant HIV-1 infected woman, because the clinical complications may be more difficult to manage during pregnancy.

HIV-1 infection in children

Pediatric HIV-1 infection is primarily due to mother-to-child transmission of the virus. The first child with HIV-1 infection was described in 1982 [20]. It soon became evident that diagnosing HIV infection in children was more complicated than in adults. The course of infection and the clinical characteristics of AIDS in children appeared also different from those in adults [21–28].

Mother-to-child transmission of HIV-1

Rate of transmission

In the absence of any intervention, the reported rates of mother to child transmission of HIV-1 ranges from 15–25% in Europe and the U.S. [29] to 25–42% in Africa [30–32]. It has been discussed if these differences, at least in part, may be due to methodological differences and proposed that standardized methods should be used to estimate rates of mother-to-child transmission of HIV-1 [33]. Nevertheless, transmission rates in Africa still appear to be twice as high as in Europe. These geographic differences are not fully understood, but may be influenced by differences in frequency of breastfeeding, other concomitant infections in the mother, as well as other differences between host and viral factors.

Timing of transmission

Transmission of HIV-1 from mother to child can occur in utero, at the time of delivery (intrapartum) and postnatally through breastfeeding.

1. In utero transmission

In utero transmission is supported by studies based on PCR and *in situ* hybridisation. Thus, HIV-1 has been detected in fetal tissues obtained during the first and second trimester [34–36]. Lewis et al. showed that embryonic blood cell precursors were infected *in vitro* by HIV-1 in 8-week fetuses [37]. An important study showed that 2 of 100 fetuses were HIV-1 infected at half pregnancy [38]. This means that

approximately 10% of all perinatally infected children become infected in early pregnancy.

2. Transmission at time of delivery

Suggestive evidence of late or intrapartum transmission came first from observations from a study on HIV-1 infected pregnant women and their offsprings. HIV-1 was easily detected in the plasma of the mothers, but not in their newborn children or in the fetuses, but easily in the child at 6 months of age [39]. Shortly after, observations from a register of twins was published, which found that the first-born twin had a twofold higher risk of becoming HIV-1-infected than the second-born twin [40]. Exposure of the fetus to virus in cervico-vaginal secretions or maternal blood is thought to play a role, although the same phenomenon was observed for twins delivered by Cesarean section. There are more recently reports indicating that the mode of delivery affects the transmission rate. Thus, delivery by elective Cesarean section prior to labour and rupture of membranes decreased [41, 42], whereas prolonged rupture of membranes (>4 hours) increased the risk of transmission [43]. Thus available data indicates that a large proportion of infections occurs at time of delivery or in late pregnancy [44], for review see [45, 46].

3. Transmission through breastfeeding

A meta-analysis of studies of transmission through breastfeeding showed the additional risk of transmission to be between 7 and 22% [47]. A recent study conducted in Durban, South Africa showed that the risk of vertical transmission was considerably lower during exclusive breastfeeding than during mixed feeding [48]. It is believed that the lower transmission rate in exclusively breastfed infants is explained by the fact that these babies develop a healthier gut epithelium, which acts as a viral barrier [49–51]. This is an important finding since it is the first time that the risk associated with exclusive and mixed breastfeeding has been distinguished. Infant feeding practice needs further investigation since this study was not randomized. It has also been suggested that late weaning increases the risk of postnatal transmission [52, 53].

4. Definition of in utero vs. intrapartum infection

A working definition for the classification of the timing of transmission has been proposed, which is based on the time of HIV-1 detection in the infant. If virus is detected within 48 hours of birth, an infant is considered to have been infected *in utero*,

while intrapartum infection is assumed if viral studies are negative during the first week of life, but become positive between 7 and 90 days of life [54]. If infected around the time of delivery, the incubation time from infection to becoming PCR positive appears to be around one week [44].

Table I

Factors affecting mother-to-child transmission of HIV-1

Factors		References
Viral	•RNA level in plasma	[55–57]
	•Viral genotype: minor or major viral population from the mother can be found in the infant	[58, 59]
	•Viral phenotype: NSI viruses transmitted to the infant SI viruses associated with transmitting mother	[60]
	•Viral resistance	[61]
Maternal	•CD4 cell count	[62]
	•Neutralizing antibodies	[63, 64]
	•Nutritional status	[65]
	•Clinical status	[24]
	•Behavioural factors	[66, 67]
	•Antiretroviral treatment	[68, 69]
Obstetrical	•Prolonged rupture of membranes more than 4 hours	[70, 71]
	•Mode of delivery	[42, 72]
	•Intrapartum haemorrhages	[66]
	•Obstetrical procedures	[43, 62]
	•Invasive fetal monitoring	[73]
Fetal	•Prematurity	[74]
	•Genetic factors	[75–77]
Infant	•Breastfeeding	[47, 78]
	•Immature immune system	[79]

Factors affecting mother-to-child transmission

Transmission of HIV-1 from mother to child is affected by a number of factors, of which many have not been fully elucidated. These can be divided into viral, maternal, obstetrical, fetal and infant factors (Table I). Here we are going to consider the influence of the viral genotype and phenotype on transmission of HIV-1 from mother to child.

Co-receptor usage and genetic subtype of HIV-1

Genetic variability is one of the major characteristics of HIV-1. In fact, HIV-1 has been described as a quasi-species, which consists of a cloud of closely related but distinct genetic variants around a central point ("the master-variant") [80]. HIV-1 can be regarded as a pool of genetic variants, from which pre-existing variants with favourable characteristics can be selected providing a potential for rapid adaptation.

At present, three HIV-1 groups have been identified: M (main), O (outlier) [81], and N (non-M-non-O) [82]. Group M viruses cause the global HIV-1 epidemic, whereas group O and N viruses occur at low frequency mainly in certain regions of Central Africa. Group M is divided into nine subtypes denoted A, B, C, D, F, G, H, J and K [83–85]. Subtypes E and I have been dropped because they turned out to be recombinant viruses [83]. The genetic distances within and between subtypes depend both on the region of the genome studied and the method used to measure the genetic distances. Generally members of the same subtype differ by less than 10% and those of different subtypes by more than 10% in the gag gene. Considering variable region 3 (V3) of the viral envelope, variation within an infected individual may be as high as 10–15%, between individuals carrying virus of the same genetic subtype about 30%, and different subtypes may differ more than 50% in nucleotide sequence [86].

Biological variability is another major characteristic of HIV-1. Primary HIV-1 isolates can be divided into different biological phenotypes according to specific *in vitro* properties, such as replicative and syncytium inducing capacities and cellular tropism [87–89]. The discovery that chemokine receptors may function as co-receptors when HIV-1 enters cells [90–93] was the missing link to our understanding of the molecular background of HIV-1 biological variation. Thus, the chemokine receptor CXCR4 serves as the major co-receptor for viruses earlier called rapid/high or syncytium inducing (SI), while CCR5 serves as the major co-receptor for slow/low or non-syncytium inducing (NSI) viruses. This allowed the introduction of a unifying nomenclature that classify HIV-1 isolates on the basis of their co-receptor usage [94]. The issue of biological variation and co-receptor usage has been dealt with in an earlier article [95].

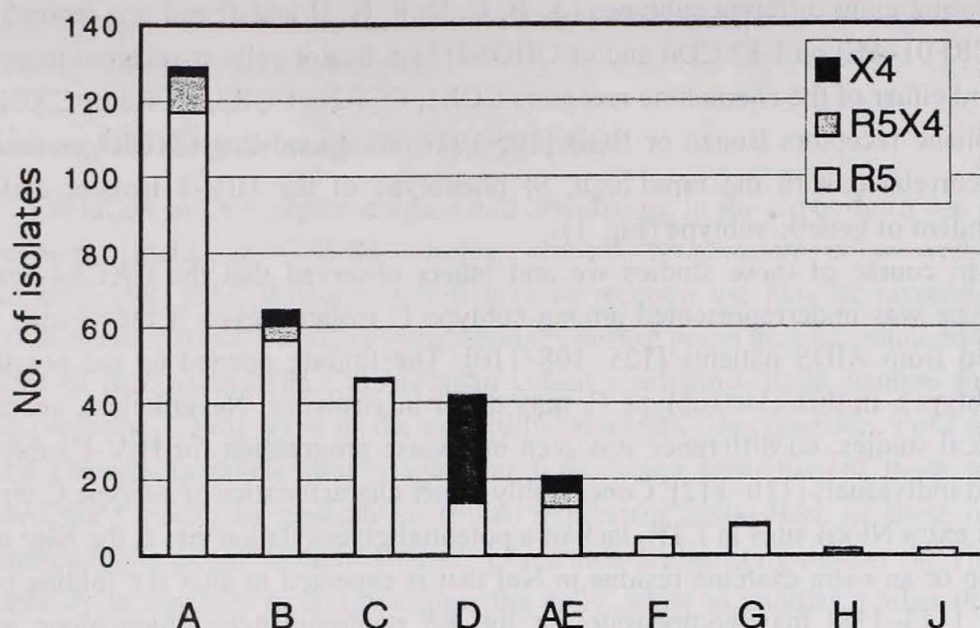


Fig. 1. Co-receptor usage of 322 virus isolates from plasma or PBMC representing nine different genetic subtypes. Several isolates were obtained from the same patients. Co-receptor usage was determined by scoring for syncytium formation, expression of green fluorescent protein, GFP (in the GHOST assay) and HIV-1 p24 antigen production. Both panels of indicator cells were kindly provided by Dr. Dan L. Littman at the Skirball Institute for Biomolecular Medicine, New York University, but are now available from the NIBSC, AIDS Reagent Programme, MRC, UK and the NIH AIDS Research and Reference Reagent Programme, USA

Several studies have demonstrated that the severity of immunodeficiency *in vivo* correlates with the biological phenotype of virus isolates *in vitro* [87, 96–98]. Following the discovery of co-receptors, it could be pinpointed that, in analogy with the previously described phenotypic patterns for HIV-1 of subtype B, viruses using CCR5 (R5 viruses) predominate in early stages of HIV-1 infection, while X4 and dual-tropic R5X4 variants appear at late stages [99]. Also, among subtype B infected mothers those with CXCR4-using virus were at higher risk of transmission of HIV-1 to their children than mothers harbouring R5 virus [60] and unpublished. The importance of the co-receptors *in vivo* was further reinforced by studies on polymorphisms in genes coding for the co-receptors and their ligands. The most important polymorphism occurs in the CCR5 gene, CCR5 Δ 32 [100–103], a deletion that causes a defect membrane expression of CCR5. Consequently, homozygosity for CCR5 Δ 32 protects against infection with HIV-1 using CCR5 for cell entry. Heterozygotes are not protected against infection but show a slower rate of HIV-1 disease progression [104].

We were interested in the question whether different genetic subtypes differ in co-receptor usage and thereby may differ in important biological properties, such as virulence and transmissibility. Overall we have tested 322 primary HIV-1 isolates

representing eight different subtypes (A, B, C, D, F, G, H and J) and one recombinant type (CRF01-AE) on U87.CD4 and/or GHOST(3) indicator cells engineered to express CD4 and either of the chemokine receptors CCR1, CCR2b, CCR3, CCR5 or CXCR4 or the orphan receptors Bonzo or BOB [105-107]. We found that CXCR4 co-receptor usage correlated with the rapid/high, SI phenotype of the HIV-1 isolates and was independent of genetic subtype (Fig. 1).

In course of these studies we and others observed that the CXCR4-positive phenotype was underrepresented among subtype C isolates, even if the viruses were obtained from AIDS patients [105, 108-110]. The finding opened up the possibility that subtypes, in this case subtype C, may differ in virulence. Nevertheless, according to clinical studies, no difference was seen in disease progression for HIV-1 subtype C infected individuals [110-112]. Conceivably, other characteristics of subtype C viruses, such as extra NF κ B sites in LTR, lack of a potential glycosylation site at the base of the V3 loop or an extra cysteine residue in Nef that is expected to alter the folding of the protein [113-118] may compensate for the R5 predominance, which alone would reduce virulence.

Subtype C had a low prevalence in the early phase of the HIV pandemic but is now spreading rapidly worldwide. At present, HIV-1 subtype C has been estimated to account for more than 50% of HIV-1 infections worldwide (<http://www.unaids.org>). In the context of mother-to-child transmission it is important to remember that in the Swedish material among 11 mothers with infected children, HIV-1 of subtype C was not overrepresented. Six different subtypes were present in this group and two of these were subtype C [119]. In another study examining the placental barrier [107], among nine HIV-1 infected mothers with uninfected children, four mothers carried subtype C virus. From two of the mothers virus with R5 and R5X4 phenotype could be isolated at delivery. This material is small, nevertheless no difference to other subtypes could be detected.

Virus isolates were also tested for usage of the orphan receptors Bonzo/STRL33/TYMSTR and BOB/GPR15 on GHOST(3) cells [106]. Among the Cameroonian women [106], we found that subtype A isolates (by envelope sequence) from five pregnant women and one child could use Bonzo in addition to CCR5. Bonzo has been shown to support efficient cell-cell fusion by a wide variety of SIV env proteins [120], but was inefficient at mediating infection by HIV-1 isolates [120, 121]. In addition to our findings, only isolates from one mother-infant pair infected with subtype B could use Bonzo as co-receptor in the GHOST cell system [122, 123]. BOB could also be used as co-receptor by some isolates in a panel representing five different genetic subtypes (A, B, C, D and F) [124]. Our data suggest that some HIV-1 isolates

may use Bonzo or BOB for productive infection *in vitro*, but the *in vivo* significance of this finding remains to be established.

Evolution of co-receptor usage during pregnancy

Evolution in co-receptor usage, i.e. a broadening in the capacity to use several co-receptors, including CXCR4, during clinical progression is a well-known phenomenon [99, 125]. Whether evolution of co-receptor use may be favored by the particular hormonal and immunological changes during pregnancy has since long been a matter of discussion. The Cameroonian cohort consisting of 28 women followed during pregnancy [106], gave us the possibility to address this question. Four mothers showed changes in co-receptor usage over time. Virus from two of these mothers acquired the capacity to replicate in Bonzo expressing cells. Both of these mothers transmitted the infection to their children. In one non-transmitting mother the virus lost the capacity to replicate in Bonzo expressing cells, while in another mother the virus showed fluctuations in its capacity to use CCR3. Comparison of the Cameroonian subtype A viruses with subtype B viruses (from Italy) showed that, in both cases, among transmitting mothers dual- or multitropic viruses were more frequent than in the group of non-transmitting mothers (Table II). While multitropism with subtype B involves in all cases CXCR4 use, subtype A viruses use Bonzo (in combination with CCR3 and/or CCR1). Taken together, 36.8% of transmitting mothers and 16.2% of non-transmitting mothers harbour multitropic viruses (Fischer exact test, $p=0.007$). The data suggests that presence of multitropic virus in the mother may increase the risk of HIV-1 transmission to the child.

In what way could the described changes in co-receptor use of HIV-1 be related to pregnancy? Alterations in certain cytokines and chemokines have been observed during pregnancy, which conceivably could modify HIV-1 replication [126]. In particular, IL-10 production, which has been reported to down-regulate HIV-1 replication [127], increases during pregnancy. Consequently, IL-10 and other cytokines may influence the milieu of the virus and thus favour growth of certain virus variants. Furthermore, all published Bonzo-using HIV-1 isolates that we are aware of, have been isolated from pregnant women and their infants. In this context, it is interesting to note that, expression of Bonzo mRNA is restricted to lymphoid tissue, PBMC and placenta [128–130]. Thus, trophoblasts from early placentae have been documented to express CCR5, CXCR4, Bonzo and GPR1 [131], whereas term trophoblasts do not express CXCR4, CCR5, CCR3, CCR2b or Bonzo [132]. Furthermore, there is evidence of bidirectional traffic of leukocytes across the placenta during pregnancy [133]. Naïve CD45RA⁺/CD62L⁺ cells that express Bonzo are much more abundant in cord blood than in adult peripheral blood [134] and between these naïve cells and maternal blood

may be a route of infection. Clearly, much more work is needed to clarify if pregnancy favours the outgrowth of virus with certain biological properties.

Table II

Comparison of co-receptor use of HIV-1 env subtypes A and B

Genetic subtype	Mother	No. of mothers	Biological phenotype close to delivery	
			R5 only	Dual/multitropic
A	Transmitting	4	2	2 R5 Bonzo
	Non-transmitting	18	14	2 R5Bonzo 2 R5R1
B	Transmitting	15	10	3 R5X4 2 R5R3X4
	Non-transmitting	19	17	1 R5X4 1 R5R3X4

Interventions reducing mother-to-child transmission/ethical aspects

In industrialized countries

The ACTG076 study, which was published in 1994 [68], showed that zidovudine prophylaxis significantly reduces mother-to-child transmission of HIV-1 by approximately 2/3, from 26 to 8%. Consequently, zidovudine treatment has been recommended from gestational week 26. At delivery, intravenous zidovudine is given. The child is also treated from birth and during 6 to 8 weeks. Blanche et al. reported a possible adverse effect, i.e. mitochondrial dysfunction, in zidovudine-exposed children [135]. However, this has not been confirmed in the trials from the United States [136]. Nevertheless, this underlines the potential risk of giving a toxic drug to children, who would remain uninfected in the majority of cases also without treatment. When zidovudine prophylaxis is given together with elective Cesarean section the transmission risk is reduced to less than 2% [42].

In resource poor settings

UNAIDS has recognised that pregnant women have a right to free HIV testing and, if they are found to be positive, to proper counselling (VCT) [137]. Numerous clinical trials using short-term treatments have shown very promising results, especially HIVNET012 a randomised trial of the antiretroviral drug, nevirapine given as a single dose [69]. Nevirapine lowered the risk of HIV-1 transmission during the first 14–16

weeks of life by nearly 50% in a breastfeeding population. This simple and inexpensive regimen could decrease mother-to-child transmission of HIV-1 in less-developed countries.

Ethical aspects

The Declaration of Helsinki is a statement on research ethics, which was written on the initiative of the World Medical Association in 1964. The goal of the declaration is to protect the subjects taking part in biomedical research from abuse and exploitation [138]. Some clinical trials on mother-to-child transmission of HIV-1 have been argued to be in conflict with the Helsinki declaration [139]. After the publication of ACTG076 trial in 1994, all placebo-controlled clinical trials can be considered to be unethical, but nevertheless such studies have been continued because they give rapid and valid assessment of an alternative drug regimen to prevent vertical transmission [139, 140]. In order to avoid this type of problem, journal editors should routinely require that every paper reporting results of medical research contain a section on ethical considerations and clearance.

The placental barrier

Structure and function of the placenta

The placenta is a fetal organ of exchange, interposed between the fetal and maternal circulations [141]. The basic functional unit of the placenta is the chorionic villus. Chorionic villi are simple structures that have a core of loosely arranged mesenchyme, which is covered by a double layer of trophoblastic cells (Fig. 2). Hofbauer cells, fibroblasts, lymphocytes, macrophages and fetal capillaries are scattered within the mesenchyme. The inner layer of the trophoblast is made up of cuboidal cells called cytotrophoblasts, which generate and maintain the outer layer. The outer layer of trophoblastic cells is termed the syncytiotrophoblasts and is a continuous multinucleated epithelium in direct contact with maternal blood. The placental barrier which separates the maternal and fetal blood systems is composed of the two cell layers of trophoblasts, the connective tissue of the chorionic villi and the endothelium of the fetal villous vessel. The structure of the placenta is established in the first trimester and has acquired a definite form by the end of the fourth month. As the placenta grows and ages, certain histological changes are suggestive of an increase in the efficiency of transport to meet the growing fetal metabolic requirements. Such changes involve a decrease in thickness of the syncytiotrophoblastic layer, a partial disappearance of the

cytotrophoblasts, a decrease in stromal volume, and an increase in the number of capillaries and their approximation to the syncytial surface.

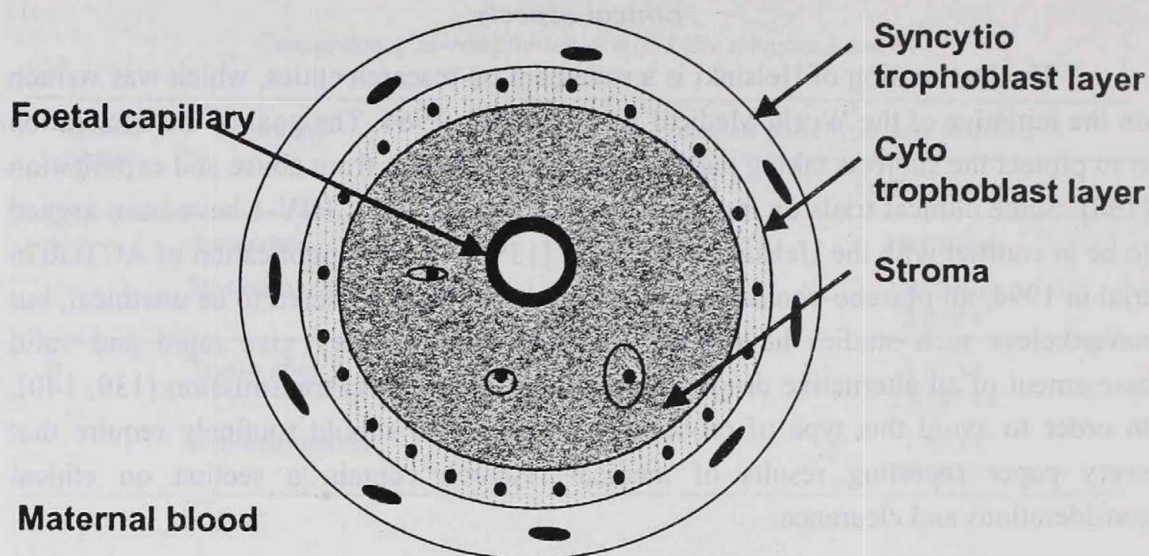


Fig. 2. Schematic view of a chorionic villus

Trophoblasts are the fetal tissue directly in contact with maternal blood. Class II MHC antigens are absent from trophoblasts at all stages of gestation, which is important for maternal acceptance of the fetus. The only Class I antigens expressed in human cytotrophoblasts is the nonpolymorphic HLA-G [142]. HLA-G expression may be crucial for invasion of the trophoblasts in the endometrium-decidua by helping to avoid killing by maternal NK-like cells (large granular lymphocyte). The syncytiotrophoblast layer expresses neither HLA class I nor HLA class II antigens [143].

The placenta in HIV-1 infected women

Upon macroscopic examination, the placenta in HIV-1 infected pregnant women often appears normal. Increased placental weight has been observed, but may not be directly related to HIV-1 infection [144]. Chorioamnionitis is the only histopathologic abnormality found with increased frequency, 43% in HIV-1-infected mothers vs. 20% in an uninfected control group [144, 145]. No abnormalities have been reported in the villous architecture of the placentae from HIV-1-infected mothers.

Immunohistochemical techniques and *in situ* hybridisation have been used in the evaluation of HIV-1-infected placentae from mothers without antiretroviral therapy. The results are contradictory. Expression of HIV-1 p24 antigen and presence of HIV-1 nucleic acid has been seen in trophoblasts from early and term placentae [37, 146, 147]. Hofbauer cells have also been found infected [37, 146, 147]. However, in these studies, the cell types were distinguished by morphology only, which makes identification uncertain. Other studies did not detect viral proteins in early and term placentae [148, 149]. Electron microscopy has detected retroviral particles, but not budding viruses [144]. These findings could be related to endogenous retroviruses [150] and need further investigation. In all cases, HIV-1 expression in the placenta did not correlate with HIV-1 infection of the fetus.

Possible mechanisms of *in utero* transmission of HIV-1 from mother to infant

Several different possible scenarios have been proposed and intensely discussed (see Fig. 3 and below). An important basis for these discussions is the fact that the virus population is genetically quite homogeneous early after transmission and that virus with R5 phenotype is much more common than virus with X4 or R5X4 phenotype [151, 152]. Many researchers have interpreted these findings as a sign of selection for virus with certain biological properties (i.e. R5 phenotype) during or immediately after transmission. The findings and discussion concerning sexual transmission of HIV-1 are very similar, but it is unclear if there are differences in mechanisms of perinatal and sexual transmission.

HIV-1 infection of the placenta

The trophoblast and syncytiotrophoblast layer, which is the outer layer of the placenta, is directly exposed to maternal blood. It has been extensively discussed whether this direct exposure of cells to HIV-1 infected blood could infect these cells. We addressed this question by using highly purified placental trophoblasts obtained from HIV seropositive mothers undergoing antiviral therapy and tested these by PCR amplification of HIV DNA and RNA [127]. We found the immunomagnetically purified trophoblast preparations HIV negative.

The cell fractionation procedures whereby trophoblasts are obtained seem to be crucial [153] and the level of contaminating nontrophoblastic cells will strongly influence the outcome of HIV detection. Other studies have detected HIV sequences in placental cell preparations [154, 155]. These cell preparations were obtained after only one round of immunomagnetic cell depletion using a single anti-CD45 monoclonal

antibody. In contrast, we carried out immunomagnetic cell separations in three sequential steps and used specific monoclonal antibodies for each cell type [107].

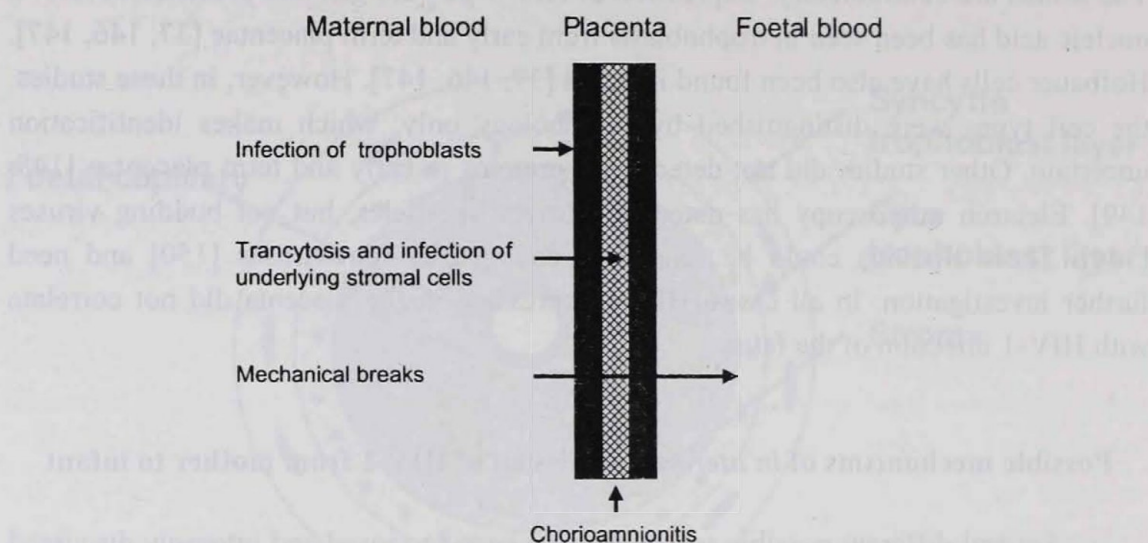


Fig. 3. Possible mechanisms for *in utero* transmission of HIV-1

HIV DNA was not detected in our immunomagnetically purified trophoblast preparations. This conclusion is supported by the finding of Kilani et al. [156] that pure placental trophoblasts resist infection by several different cell-free primary HIV-1 isolates. In fact, we were able to identify the HIV-1 DNA positive cells as CD3+ or CD14+, i.e. contaminating T-lymphocytes or macrophages, respectively. This is in line with previous studies demonstrating that non-trophoblastic placental cells carry HIV infection *in vivo* and are susceptible to infection *in vitro* [34, 149, 157, 158].

Another problem with inadequate separation techniques in these types of experiments is that the non-trophoblastic cell fraction of the placenta will consist of a mixture of maternal and fetal cells. Consequently, the demonstration of HIV infection in non-trophoblastic cells may simply be a reflection of the fact that the maternal blood in the placenta contains HIV infected CD4+ lymphocytes and monocytes. De Andreis and colleagues detected HIV provirus in more than 70% of placentae from HIV-1 infected mothers, who did not receive antiretroviral therapy [159]. They used DNA polymorphism analysis, based on identification of hypervariable regions in the human genome to rule out maternal contamination. However, this method can confirm, but not exclude, maternal contamination due to limitations in the sensitivity of detection of minor sequence variants. This means that significant contamination by HIV infected maternal blood cells may have gone unnoticed [Chamberlain, 1980 #1045]. We used two approaches to test the origin of HIV infected cells. First, the amount of HIV DNA

in each cell fraction was determined by a semi-quantitative, limiting dilution assay. For all mothers, PBMC and placental CD3+ T-lymphocytes carried similar amounts of HIV-DNA suggesting that the viral DNA was of maternal origin. Second, we determined the origin of cells in the different cell preparations by microsatellite analysis. Indeed, in our experiments we could confirm that the different non-trophoblastic placental fractions consisted of mixtures of maternal and fetal cells.

While CD3+ T-lymphocytes were regularly HIV positive, DNA in CD14+ cells of the placenta could be detected in three cases only. These findings are in line with a report of relative refractoriness of the placental macrophages to *in vitro* infection with primary isolates [160]. Taken together, our results and those of others strongly indicate that the trophoblastic barrier remains uninfected in a majority of HIV-1 infected pregnant women [34, 156].

All mothers in our study were receiving zidovudine therapy according to the ACTG076 protocol and some mothers in addition received other reverse transcriptase inhibitors. We cannot exclude that the absence of HIV-1 infection in the trophoblasts in our study may have been influenced by the antiretroviral therapy. However, the antiviral therapy was clearly sub-optimal in three mothers who had relatively high plasma HIV RNA levels. This suggests that the antiretroviral therapy is not the sole explanation for the absence of HIV DNA in the trophoblasts. It is also important to mention that all children born by mothers in our study appear to be uninfected. Thus, we cannot formally exclude the possibility that trophoblasts may be infected in pregnancies which result in HIV transmission to the fetus.

Our results indicate that the trophoblastic barrier remains uninfected in full-term placentae of HIV-seropositive mothers undergoing antiviral therapy. Possible other mechanisms of *in utero* transmission will be discussed in the followings. A schematic illustration of possible mechanisms involved in *in utero* transmission of HIV-1 is presented in Figure 3.

Selective infection or transport through the placenta?

The placenta is an obvious barrier between the mother and the child that could potentially select for virus with certain biological properties. Virus could pass the intact placenta either by some type of transport or by direct infection of the cells in the placenta. Our results clearly argue against infection of trophoblasts [107]. This is in line with the receptor expression pattern on trophoblastic cells. Trophoblasts do not express CD4 (mRNA or cell membrane antigen) [161–163]. Trophoblasts from early placentae have been shown to express CCR5, CXCR4, Bonzo and GPR1 [131], whereas no expression of CXCR4, CCR5, CCR3, CCR2b, Bonzo has been detected on term trophoblasts [132]. Thus, if trophoblasts are infected they would have to be

infected through a CD4-independent pathway, such as via Fc-receptors. The presence of Fc-receptors on the trophoblast is well documented [164].

Trancytosis of cell-bound, but not cell-free, HIV-1 through an artificial trophoblastic barrier has been described *in vitro* [165]. Once transported across the trophoblastic layer, virus could potentially spread to stromal cells, such as Hofbauer cells, which have been shown to sometimes be infected in placentae of HIV-1-infected mothers. The Hofbauer cells are placental macrophages of probable fetal origin that express CD14 and CD4, but not CCR5 or CXCR4 [160, 166, 167]. Placental macrophages have been shown to be permissive for infection with laboratory strains [158, 168, 169], but refractory to infection with various primary isolates [160].

Non-selective transmission through the placenta: mechanical breaks in the placental barrier or chorioamnionitis

A second hypothesis, which we favour states that transmission through the placenta is non-selective, but that there may be selective outgrowth of R5 virus in the infant after transmission. We have been studying co-receptor usage of isolates from 11 Swedish HIV-1 infected mother-child pairs [119]. The results showed that there was a predominance of R5 virus early after perinatal infection. A CXCR4-switch was later documented in three of the children. Importantly, the mothers of two of the children that displayed a CXCR4-switch also carried CXCR4 using virus, while all other mothers carried R5 virus. The fact that two of three children with late CXCR4-switches had mothers with documented CXCR4 using virus suggests that these variants were in fact transmitted, but that they were suppressed in the children during the first years of life [119].

The failure of the placenta to maintain absolute integrity of the fetal and maternal circulations is documented by numerous reports on the passage of cells between mother and fetus in both directions [141]. It has been reported that leukocytes from the fetus can survive maybe also divide in the mother; thus leukocytes with Y-chromosomes have been identified in blood of women up to five years after they have given birth to a son [170]. It has also been shown that maternal leukocytes can pass into the fetus [171]. The mechanism of passage of the cells is poorly understood. Breaks in the continuity of the syncytiotrophoblastic surface can occur from early pregnancy to delivery, and these defects are repaired by fibrin. Thus, it is very possible that HIV-1-infected cells may also cross from mother to child through physical breaks in the placenta [172].

As mentioned, chorioamnionitis is the only histopathologic abnormality, which is regularly found in placentae of HIV-1-infected mothers. It is not clear whether these findings are HIV-1 specific or manifestations of other infections [145]. If

chorioamionitis is HIV-1 specific, it is possible that the fetus could become infected through an ascending route or by swallowing and aspiration of infected amniotic fluid [173].

According to the second hypothesis there is no selection of viral variants at the level of the placenta. Selection, if it occurs, would be more likely to occur in the host. We have indications that X4 and R5 virus were both transmitted from two mothers, but that R5 virus replicated in the blood compartment of the infant during the first months of life [119]. We will use molecular techniques to try to clarify if the CXCR4 variants in the infants were transmitted from the mothers or arose *de novo* in the infant by evolution from CCR5 variants.

Conclusions

With the global increase in human immunodeficiency virus 1 (HIV-1) infection in women of childbearing age, there has also been an alarming increase in the number of mother-to-child transmissions of HIV-1. Although antiretroviral therapy and Elective Caesarian section have been demonstrated to significantly decrease the vertical transmission rate of HIV-1, these interventions are not widely available in the developing world. Therefore, studies of the mechanisms of vertical transmission are important.

We have been studying genetic and biological variability of HIV-1 in prospectively followed HIV-1-infected patients, especially pregnant women, in Sweden and in Cameroon. The general rule emerging from these studies was that CXCR4 usage determines the biological phenotype (slow/low, NSI or rapid/high, SI) for all subtypes, but the frequency of CXCR4-usage may vary between HIV-1 subtypes. Notably, CXCR4 use was rare among subtype C isolates. This finding was not due to differences in clinical status, CD4 count or treatment. Likewise, subtype C infection was not overrepresented among mothers with infected children.

Earlier work with subtype B suggested that presence of multitropic virus (R5X4 or R5R3X4) in the mother may increase the risk of HIV-1 transmission to the child. We now found R5Bonzo-using virus in some pregnant HIV-1 envelope subtype A infected women from Cameroon [106]. Comparison of the Cameroonian subtype A viruses with subtype B viruses showed that, in both cases, multitropic viruses are more frequent among transmitting mothers than in the group of non-transmitting mothers (37% and 16%, respectively).

The placental barrier function was tested in nine term placentae from a cohort of pregnant women in Sweden, identified as infected by HIV-1 subtype A, B or C. All

mothers underwent antiretroviral therapy and all children remained uninfected. HIV-1 sequences were detected by PCR in the mother's blood and in enriched placental trophoblastic cell preparations. After several rounds of immunomagnetic cell separation of these mixed populations, the purified trophoblasts were overall negative. Proviral DNA was found in the non-trophoblastic placental cells, mainly T-lymphocytes, which were shown to be a mixture of maternal and fetal cells. This study indicates that the placental barrier, i.e., the trophoblastic layer, does not become HIV infected. If HIV-1 infection of the fetus occurs, it is likely to be through other routes, such as breaks in the placental barrier.

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ISOLATION AND IDENTIFICATION OF PATHOGENIC STRAINS OF *STREPTOMYCES ACIDISCABIES* FROM NETTED SCAB LESIONS OF POTATO TUBERS IN HOKKAIDO (JAPAN)*

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A relatively homogeneous group of streptomycete isolates was obtained from netted scab lesions of potato tubers collected from a potato field in Hokkaido, Japan. Based on 55 phenotypic data of 72 *Streptomyces* strains selected from these isolates together with spectral data on their soluble pigments and with data of a PCR analysis, using species specific primers, these netted scab causing pathogenic organisms were identified as *S. acidiscabies*. *S. acidiscabies* had previously been isolated from deep (common) scab lesions in the USA and reported as thaxtomin A producer. In contrast, our *S. acidiscabies* strains were not able to induce deep scab symptoms on potato minitubers in pot test, did not produce the phytotoxin thaxtomin A and did not contain the pathogenicity related gene, *nec-1*.

Keywords: netted scab, potato, *S. acidiscabies*, thaxtomin A, *nec-1*

Potato scab diseases are responsible for serious economical losses in the potato production of the developed countries. Scab pathogens attack growing potato tubers and/or other underground structures of potato plants. Two types of potato scab diseases can be distinguished: common (deep) scab and netted scab diseases. Common scab is

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characterized by erumpent or deep-pitted corky lesions on potato tubers, while netted scab diseases can be characterized by superficial reticulations on the surface of tubers. Common scab is more important in the table crop production than netted scabs because it is more prevalent and scab lesions may extend deep into the tuber tissues.

S. scabies is the predominant common scab pathogen and it is present in all potato producing areas of the world [1–4]. *S. acidiscabies* and *S. turgidiscabies* have also been reported as the causative agents of common scab disease in the USA and Japan, respectively [5–7]. Beside these species other streptomycetes have also been reported as the causative agents of common (deep), russet, netted or other types of superficial scab lesions on potato tubers [3, 8, 9].

Recently, novel types of phytotoxins, designated as thaxtomins, have been isolated and identified from potato tuber tissues attacked by *S. scabies*. Thaxtomins are cyclized dipeptides composed of N-methylated L-4-nitrotrypto-aryl and variously hydroxylated, N-methylated L-phenylalanyl moieties [10]. Thaxtomin A is the main phytotoxic compound of potato common scab-pathogenic *Streptomyces* species [11], while thaxtomin C is produced by *S. ipomoeae* the causative agent of soil rot of sweet potato [12].

Bukhalid and Loria identified a pathogenicity related gene, *nec-1* from *S. scabies* [13]. They showed a positive correlation between the thaxtomin A production of 43 strains of three common scab-pathogenic *Streptomyces* species and (with one exception) the presence of *nec-1* gene [14]. More recently, Healy et al. [15] reported on *txtAB* genes which encode a peptide synthetase required for thaxtomin A production and pathogenicity in common scab-pathogenic *Streptomyces* species.

In the area of the island Hokkaido *S. scabies* and *S. turgidiscabies* are the predominant common scab pathogens. Tanaka et al. [16] reported on the phenotypic characteristics and pathogenicity of these streptomycetes, Takeuchi et al. [17] reported on their phylogenetic relatedness, while Tóth et al. [18] reported on their thaxtomin A production. However, in the potato experimental field of Hokkaido University common scab disease occurred rarely, while netted scab was detected frequently. From the netted scab lesions of these tubers a streptomycete producing white aerial mycelium was always detected. Production of white aerial mycelium on different agar media is not a characteristic feature of *S. scabies* or *S. turgidiscabies*. To identify this novel scab pathogen, we carried out a taxonomical work on the *Streptomyces* population of these netted-scabbed tubers.

Here we report on the characterization and identification of these *Streptomyces* strains and on their pathogenicity.

Materials and methods

Isolation of streptomycetes from netted scab lesions

Netted scabbed tubers of the potato cultivar Irish Cobbler were collected from the experimental field of Hokkaido University, Japan in 1996 and 1997. Isolation of *Streptomyces* strains from scabbed tubers was carried out as described by Elesawy and Szabo [1].

Characterization and identification of our netted scab strains

Characterization of strains was carried out by using the diagnostic criteria and standard methods recommended by the ISP (International *Streptomyces* Project) [19]. For the identification of netted scab strains the species description of Lambert and Loria was used [5].

Reference strains

The following pathogenic thaxtomin A-producing reference strains were used in this study: *S. acidiscabies* 84.104 and 84.105 (ATCC 49003 and ATCC 49004) [5] and *Streptomyces scabies* var. *achromogenes* strains IV-1, IV-6, V-3, V-20, VI-3, VII-23 [20].

Plant bioassay

Pathogenicity of our *Streptomyces* strains was evaluated in the greenhouse. Minutubers generated on stem cuttings were inoculated with a spore suspension of each *Streptomyces* strains, according to the method of Loria and Kempter [21]. After five days incubation potato tubers were scored for studying the scab lesions.

Thaxtomin A and diffusible pigment production by Streptomyces strains

Oatmeal broth medium was used for studying the thaxtomin A and/or diffusible pigment production by all of our *Streptomyces* strains according to the method of Babcock et al. [22]. Briefly, the culture filtrates of strains were extracted by chloroform. The crude chloroform extracts were loaded on precoated Merck DC-Alurolle silcagel and developed in chloroform/methanol (9:1). Bands comigrating with thaxtomin A standard [18] and/or diffusible pigment standard were recorded.

Isolation and identification of a pH-sensitive, diffusible pigment of Streptomyces strain S-68 isolated by us from netted scab lesion of potato

Glycerol asparagine broth [19] was used for diffusible pigment production by our netted scab strain S-68. Isolation and purification of the main pigment compound of this strain was carried out as described by King and Lawrence [23]. FAB and FD mass spectra of this compound were obtained on JEOL JMS AX-500 and JEOL JMS SX-102 A mass spectrometers. ^1H NMR spectra were obtained for solutions in deuterated methanol and acetone with a Bruker AMX 500 spectrometer operating at 500 MHz. Chemical shifts were measured downfield from the signal of internal tetramethylsilane.

DNA isolation from our Streptomyces strains and PCR amplifications of a S. acidiscabies-specific DNA and the nec-1 gene sequences

Isolation of total DNA from our netted scab *Streptomyces* strains was carried out according to the method of Rao et al. [24]. PCR amplification of *S. acidiscabies*-specific DNA sequence containing the 16S-23S rRNA ITS region from our netted scab strains was carried out as described by Tanaka [25].

For the amplification of *nec-1* gene sequence the following primers were used: NEC-5 (f): 5'-GATGAGCGCGAACGGAAG-3' and NEC-3 (r): 5'-TTTCTCGTTATCCATATA-3'. PCR amplification was performed in a 50 μl reaction mixture (total volume) containing 60 mM Tris- SO_4 (pH 9.1), 18 mM $(\text{NH}_4)_2\text{SO}_4$, 1.5 mM MgSO_4 , primers 100 ng each, dNTP-s 300 μM each, 1 μl template DNA and 1 U Elongase Enzyme Mix (Gibco BRL). Amplification was performed with the following program: DNA denaturing at 94 $^\circ\text{C}$ for 30 sec, annealing at 50 $^\circ\text{C}$ for 1 min and elongation at 72 $^\circ\text{C}$ for 2 min for 35 cycles. PCR products were examined with agarose gel electrophoresis, using 0.8% agarose gels in TAE buffer. Gels were stained in ethidium bromide and photographed in UV light.

Results

From netted scab lesions of Irish cobbler potato tubers collected from the experimental field of Hokkaido University 350 *Streptomyces* isolates were obtained. These isolates proved to be very similar based on their cultural morphological diagnostic properties. They produced sparse, whitish aerial mycelium on oatmeal agar, yellow-brown substrate mycelium and brownish-red diffusible pigment. 72 strains were selected from them for further detailed studies. They produced well-developed white

aerial mycelium, rectus-flexibilis sporophores and smooth spores on yeast extract-malt extract-, salt starch- and Czapek-Dox agar. They did not produce melanoid pigments on tyrosin agar and on pepton-iron agar. However, on glycerol asparagine medium these strains produced a vast amount of brownish-red, diffusible pigment that turned yellow when it was acidified with 0.1N HCl and became red when it was treated with 0.1 N NaOH. These netted scab strains behaved similarly as to their physiological biochemical properties, too. These strains could utilize all ISP (International *Streptomyces* Project) carbon compounds (L-arabinose, D-fructose, D-glucose, L-rhamnose, sucrose, D-xylose and *meso*-inositol) as sole C-sources in the medium, except raffinose. From the studied nitrogen sources they utilized L-histidine, L-hydroxyproline, L-valine but they were not able to utilize L-phenylalanine or L-methionine. They could grow in the presence of 10 µg/ml thallium acetate or 4% of NaCl. These strains could grow at pH 4.0 and on agar media containing 100 µg/ml potassium tellurite. Growth did not occur in the presence of 10 µg/ml sodium azide, 1µg/ml crystal violet, 0.1% phenol or 7% NaCl. Based on these properties 70 strains out of 72 representative netted scab strains were identified as *S. acidiscabies* (Table I).

The pathogenicity of our strains was tested in the greenhouse. None of the *S. acidiscabies* strains isolated by us from netted scab lesions was able to induce common (deep) scab lesions on minitubers, while all thaxtomin A producing reference *Streptomyces* strains caused typical common scab lesions on minitubers under the same greenhouse conditions (Fig. 1).

All *Streptomyces* strains were screened for phytotoxin production. Although we were not able to detect thaxtomin A production in oatmeal broth by our netted scab strains, production of a red, diffusible pigment was always recorded. We isolated the pH-sensitive pigments of our netted scab strain S-68 and characterized them by spectral means. The main pigment compound of this strain had a mw of 338 and had identical ¹H NMR spectra with the naphthoquinone derivative produced by *S. acidiscabies* 84.104 [23]. Production of the same pigment by our other netted-scab strains verified their identity (Fig. 2).

The specific identity of the netted scab strains was checked by PCR analysis. The 836 bp, *S. acidiscabies*-specific sequence was amplified from 8 randomly selected netted scab strains and from two authentic, thaxtomin A producing *S. acidiscabies* strains (Fig. 3).

The pathogenicity related gene, *nec-1* (663 bp) was amplified from *S. scabies* var. *achromogenes* VII-23 strain and from two authentic *S. acidiscabies* strains (84.104 and 84.105). In contrast, we were not able to amplify the same sequence from our *S. acidiscabies* strains isolated from netted scab lesions in Hokkaido (Fig. 4).

Table I

Comparison of selected diagnostic properties of 70 representative *S. acidiscabies* strains isolated from netted scab lesions of potato tubers in Hokkaido with the type strain of *S. acidiscabies* (ATCC 49003)

Characteristics	Type strain of <i>S. acidiscabies</i> (ATCC 49003)	Data on 70 strains of <i>S. acidiscabies</i> from Hokkaido
Colour of aerial mycelium	White-series	White-series
substrate mycelium	Yellow-brown	Yellow-brown
Soluble pigment	Red/yellow	Red/yellow
Spore chain morphology	Rectus	Rectus
Spore surface (EM obs.)	Smooth	Smooth
Melanoid pigments	—	—
Carbon source utilization		
L-Arabinose	+	+
D-Fructose	+	+
D-Glucose	+	+
D-Mannitol	+	+
Raffinose	—	—
L-Rhamnose	+	+
Sucrose	+	+
D-Xylose	+	+
meso-Inositol	+	+
Minimum pH for growth	4.0	4.0
Growth in the presence of		
Thallium acetate (100 µg/ml)	—	—
Thallium acetate (10 µg/ml)	+	+
Sodium chloride (7%, wt/vol)	—	—
Christal violet (100 µg/ml)	—	—
Penicillin G (10 IU/ml)	+	+
Phenol (0.1%, wt/vol)	+	—
Streptomycin (20 µg/ml)	+	+

Discussion

Due to the contradictory data on potato scab-pathogenic *Streptomyces* species (as to their various characteristic diagnostic features, the different scab symptoms they induce and the different ecological conditions under which they operate) their taxonomical status was obscure for a long time [1, 3]. With the cumulating data on streptomycetes causing common (deep) scab disease and with the application of molecular biological technics in microbial systematics, it became clear that *S. scabies* is the predominant common (deep) scab pathogen all over the world [6, 17]. However *S. acidiscabies*, *S. turgidiscabies*, *S. albidoflavus*, *S. rochei*, etc., are also scab

pathogens (common or netted scab inducers) [8], but their geographical distribution is restricted.



Fig. 1. Common (deep) scab symptoms on potato minitubers inoculated with *S. scabies* var. *achromogenes* VII-23 strain (left) and with thaxtomin A producing, *nec-1* positive *S. acidiscabies* 84.104 reference strain (centre) compared with a minituber inoculated by *S. acidiscabies* S-71 strain isolated from netted scab in Hokkaido (right)

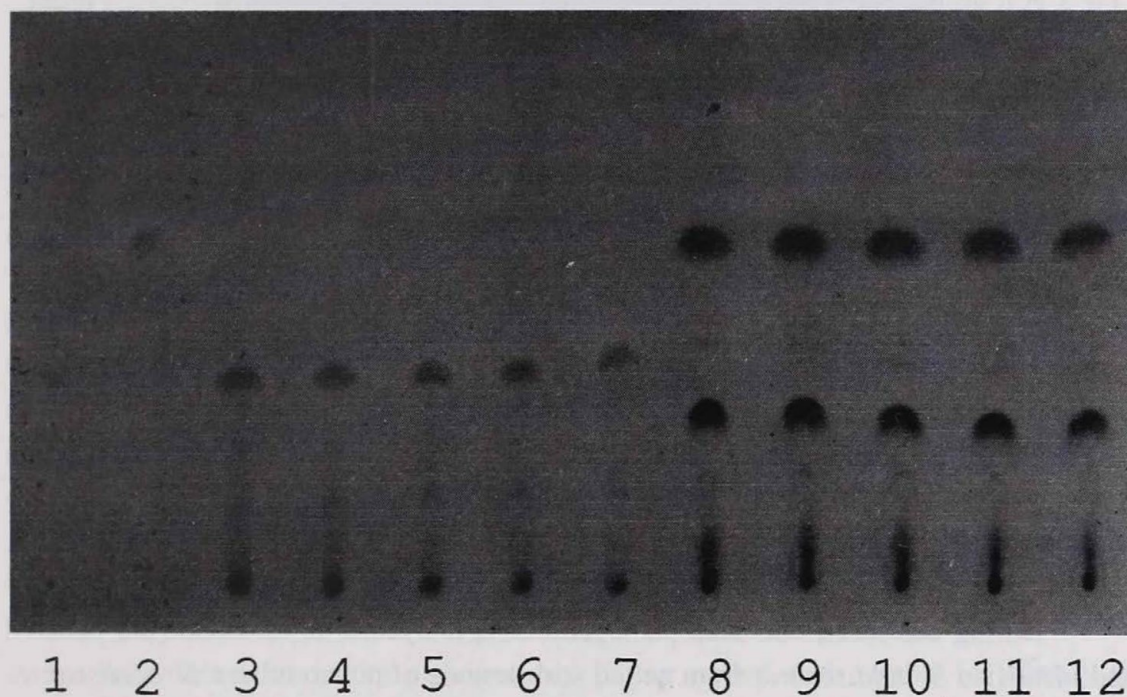


Fig. 2. Pigment and thaxtomin A production of *Streptomyces* strains. Lane 1, Thaxtomin A standard, lane 2, diffusible pigment standard., lanes 3–7, *S. scabies* var. *achromogenes* IV-1, IV-6, V-3, V-20, VI-3 strains isolated from common (deep) scab lesions, lanes 8–12, *S. acidiscabies* strains isolated from netted scab lesions in Hokkaido



Fig. 3. PCR amplification of a *S. acidiscabies*-specific DNA sequence (836 bp) from *Streptomyces* strains. Lane 1, λ DNA digested with *Hind* III, lanes 2–5, and 14–17 belong to *Streptomyces* strains isolated from netted scab lesions, lanes 6–7, *S. acidiscabies* 84.104 and 84.105 reference strains and lanes 8–13, *S. scabies* var. *achromogenes* IV-1, IV-6, V-3, V-20, VI-3, VII-23 strains

Demonstration of the role of phytotoxins thaxtomins in the etiology of potato common scab disease and thaxtomin A production by *S. scabies*, *S. acidiscabies* and *S. turgidiscabies* led to the plausible explanation how these taxonomically distinct pathogenic species are able to cause the same disease [10, 11]. The same pathogenicity mechanism suggested the possibility of horizontal transfer of pathogenicity genes among scab pathogenic streptomycetes. Molecular-biological studies proved the determinative role of thaxtomins in scab pathogenesis [15] and provided evidence for the horizontal gene transfer of pathogenicity genes among common scab inducers [14, 15].

During our studies on scab pathogenic streptomycetes in Hokkaido we isolated and identified *S. acidiscabies* from netted scab lesions of potato tubers. *S. acidiscabies* was described in the US as a common (deep) scab pathogen [5, 26] and reported as thaxtomin A producer [11]. In contrast our *S. acidiscabies* strains were not able to induce common scab disease. Presumably, this is due to the genetical differences between the American and Japanese *S. acidiscabies* strains. In fact our *S. acidiscabies*

strains did not contain the pathogenicity related gene *nec-1* moreover they did not produce thaxtomin A.

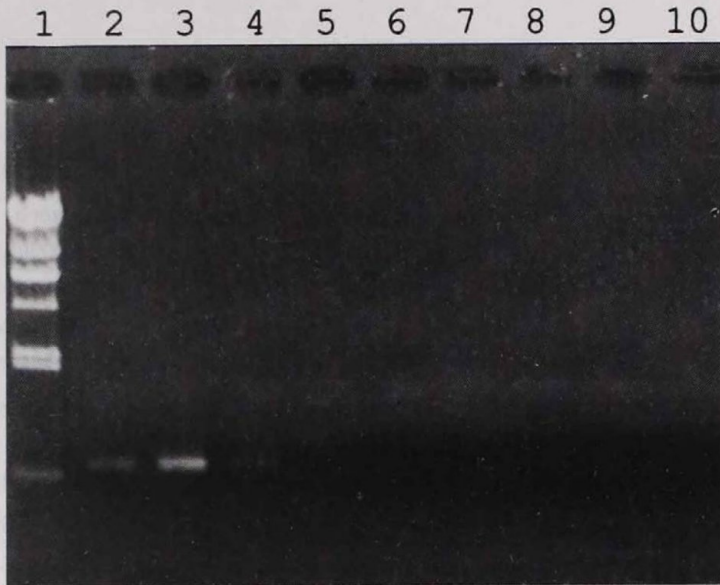


Fig. 4. PCR amplification of *nec-1* gene (663 bp) from *Streptomyces* strains. Lane 1, λ DNA digested with *Hind* III, lane 2, *S. scabies* var. *achromogenes* VII-23 strain., lanes 3 and 4 *S. acidiscabies* 84.104 and 84.105 reference strains and lanes 5–10, *S. acidiscabies* strains isolated from netted scab lesions of potato in Hokkaido

Our results agree with those of Kreuze et al. [27]. These authors reported on non-pathogenic *S. turgidiscabies* strains isolated in Finland, which were *nec-1* negative. Although they did not present data on the thaxtomin A production of their non-pathogenic *S. turgidiscabies* strains, it is very probable that non-pathogenic variants of potato scab inducers are frequent in cultivated soils and these streptomycetes can be transformed into pathogenic ones (netted or common scab inducers) under appropriate soil conditions (via horizontal gene transfer). In the future we will further study the pathogenicity of our netted scab strains to identify those factors which are specifically involved in netted scab formation and make attempts to transform some of these strains with pathogenicity genes described in plant pathogenic streptomycetes.

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FIFTY YEARS OF DYSENTERY RESEARCH AT THE PÉCS UNIVERSITY*

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Károly Rauss was appointed as head of the Department of Public Health of the Elisabeth University of Pécs in 1946. Professor Rauss's career had started working with Professor Hugó Preisz in Budapest. During his residency years he was already appointed to the Department of Bacteriology chaired by Lovrekovics at the National Institute of Public Health. In this institution – as in all organizations affiliated with the Rockefeller Institute – the state of art diagnostic work together with research focusing on problems derived from everyday medical and public health practice was considered as to be of primary importance. Stimulated by this scientific environment Rauss's interest turned towards enteric pathogens. In cooperation with Lovrekovics he developed a typhoid vaccine containing a trichloroacetic acid extract of the pathogen adsorbed to aluminum hydroxide. This vaccine was introduced in 1938 when ca. 6–8000 enteric fever cases were registered in Hungary annually. The vaccination, supported by the public health work concerning carriers, eventually led to the eradication of the disease in Hungary.

Keywords: dysentery research, Pécs University

Although while still in Budapest Rauss became interested in pathogens causing bacillary dysentery. His work with this pathogen developed after moving to Pécs. First, in 1947, he started studying the antigens of *Shigella flexneri*. These studies led to the development of the antigenic scheme in which he already distinguished group- and type-specific antigens. As revealed at the beginning of the 1970s the group antigens are

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coded by the *rfb* loci of the chromosome, while the type antigens are the results of lysogenic conversion [1, 2, 3].

His aim was to develop an effective anti-dysentery vaccine. So, parallel to the efforts to describe the antigenic scheme of this pathogen, investigations on the immunogenicity and protectivity were also launched. The mouse model used allowed to evaluate the specificity of protection. The results stressed the importance of knowing the antigenic structure: homologous protection was easily attainable, but some level of heterologous protection was also achieved due to group specific antigens [4]. Passive protection, using hyperimmune animal sera, as well as sera from patients, was also shown in the model. The protective efficacy of human sera started to decrease ca. 3 months after the infection and diminished 6 months later [5, 7].

As to develop a suitable method to prepare an acellular vaccine it was shown that with trichloroacetic acid 65% of the somatic antigen could be extracted. However, although the extract was protective, it retained a considerable part of the endotoxic activity of the LPS molecule [6].

After this period of time – until the second half of the 1970s – an important direction of research at the Department (by that time named Department of Microbiology) has focused on vaccine development. In the following sections of this review we will attempt to follow the path of vaccine research and only briefly refer to results of more basic studies.

The project to develop a successful anti-dysentery vaccine was carried out in close cooperation with the "HUMAN" Vaccine Production and Research Institute in Budapest. At the beginning it was evident that a polyvalent vaccine is needed which – according to the local epidemiological situation – should contain several *S. flexneri* types together with *S. sonnei*. Obviously, this may result in a high endotoxin load, so the tolerability of the vaccine was of primary concern. In case of *S. sonnei* we had to face another problem, too. "Phase-switch" from phase I to II was known to occur with very high frequency resulting in non-protective phase II variants [8]. It turned out that the trichloroacetic acid extraction procedure extracts the phase I antigen only, thus it was clear that a relatively stable phase I strain would be advantageous for antigen preparation.

The cooperation with the HUMAN Institute soon resulted in an important development: the use of the preformed $\text{Al}(\text{OH})_3$ gel developed by Réthy in the vaccine instead of the original, *statu nascendi* $\text{Al}(\text{OH})_3$ adjuvant. In some sense it was a detoxification called "functional detoxification" by Réthy [9]. In mice the original adjuvant resulted in a 2.5-time, while the preformed one, an 8-time reduction in toxicity which was accompanied by a 10 times increase in immunogenicity. This feature of this adjuvant was also retained in a typhoid-dysentery-tetanus antigen

combination. Some 10,000 volunteers in three age groups were vaccinated with this trivalent vaccine. The side-effects were moderate. In passive protection studies in mice the anti-shigella activity of the sera of vaccines equaled to that observed with samples from dysentery patients. It should be noted that this trivalent vaccine showed an efficacy against typhoid and tetanus equal to the typhoid-tetanus combination [10]. Systematic studies revealed the optimal intervals between booster vaccines and that the various components of the polyvalent vaccine had no negative modulating effect for other antigens [11, 12].

The capacity of shigella Boivin extracts to inhibit passive hemagglutination correlated well with the protectivity in the mouse model. Thus, using the micro-method developed by Takátsy a simple and quick method could be used to evaluate the antigenicity of various extracts [13].

It was of considerable importance to know whether an effective level of protection against carrier state could be achieved or not. It is known that during epidemics the rate of transient carriers shedding the pathogens exceeds several times the rate of patients. Chronic carrier state has also been described with or without clinical exacerbation. Investigating serum samples of carriers we found that sera of transient carriers did not, while chronic carriers did contain protective anti-shigella antibodies in high titers. This situation was rather similar to that found in typhoid carriers [14].

The first publications describing copro-antibodies appeared at the end of the 1950s. Our interest turned towards the possibilities of oral vaccination against bacillary dysentery. Following oral vaccination of mice with very high doses we could successfully detect these antibodies in the animals' gut, together with proving their protective capacity [15].

Although the parenteral mouse model using hog gastric mucin as a potentiator for the infection was suitable to measure antigen-protectivity it has always been obvious that this system was far from being relevant for a human enteric infection. Several systematic studies were published showing that after eradicating the normal flora enteric pathogens were able to induce infection even if administered in low doses. This antagonistic effect of the normal *E. coli* flora was also observed in our laboratory. Using shigella strains as infective organisms we developed a "mouse shigella model". The normal enteric flora was eliminated by high doses of streptomycin, thereafter animals were infected with shigella strains – and kept under sterile conditions – developed a long-term carrier state. Shigellae were invasive in the gut, usually reaching the lamina propria, occasionally, however, even the regional lymph nodes [16]. By preventing the development of this carrier state it was possible to measure the

effectiveness of active immunization or the protectivity of serum-, or copro-antibodies [17, 18].

A hypothesis was also developed to explain the protective role of the normal *E. coli* flora. *In vitro* experiments using hog gastric mucin suggested that *E. coli*, shigella and salmonella strains compete for a common receptor for which a possible ligand may be located in their outer membrane [19].

Histological studies on the mouse shigella model showed that shigella cells adhere to the mucin produced by the goblet cells. The invasion and intracellular multiplication in the epithelial cells together with the degeneration and denudation of the epithelial surface was also noted. The lack of clinical signs in infected animals could possibly be explained with the known elevated tolerance of mice to endotoxin [20].

At the end of 1960s we gave up the goal to develop a parenteral, polyvalent anti-dysentery vaccine – partly due to the lack of epidemiological data. In the civil population only the vaccination of extremely large number of people would have provided sufficient amount of data to allow scientific analysis. The military authorities – although keen to introduce a vaccine – did not allow to establish a placebo group and tended to keep correct figures as a secret. The possibility to joint a Central-American typhoid vaccine study was raised but was not supported by the Hungarian authorities.

That time, our experiments with the mouse shigella model and oral vaccination run parallel with pathogenicity studies. Based on the light refraction features of colonies we succeeded to distinguish between virulent and avirulent clones. Virulent clones of *S. flexneri* selected as low as 5 CFUs were enough to induce the long-term carrier state in the mouse model. According to Formal three basic steps could be distinguished in the pathogenesis of bacillary dysentery: (1) Multiplication in the gut; (2) Invasion of epithelial cells; and (3) Intraepithelial multiplication. This was the time when the possibility to vaccinate with live attenuated strains was raised. Since previously we successfully used the intravenous inoculation of chicken embryos [21] with success to measure endotoxic activity attempts were made to use this model to select avirulent clones. We found that strains avirulent in the Serény test were also non-pathogenic in this latter model. The intravenous injection of chicken embryos was combined with injection into the allantoic cavity of the eggs. Clones selected in this way were grouped into three categories: virulent clones, avirulent ones, and ones killing the embryos only by intravenous injection, but not when inoculated into the allantoic cavity (presumably non-invasive derivatives), respectively [22].

Using the mouse shigella model immunization experiments were carried out with various live and killed avirulent clones and with their trichloroacetic acid extracts. The live vaccine was not significantly more effective than the other ones, and –

particularly due to safety considerations – the acellular vaccine was considered optimal [23].

The acellular oral vaccine was used in human volunteers, as well. First, it was used as a booster vaccine for those who previously had received a parenteral primary immunization. In cooperation with the HUMAN Institute the Boiven extract was precipitated with ethanol and lyophilized, and tablets were formed using *saccharum lactis vehiculum*. The oral vaccine was completely free from any side effects and with a dose exceeding 30 times the one used for parenteral immunization protective serum level was maintained [24]. Later it was also shown that immunity could also be induced with oral priming and maintained by booster doses in 4 weeks intervals [25].

Since the chances to carry out large-scale human vaccine studies with data available for scientific analysis did not improve we established a cooperation with researchers at the monkey farm in Sukhumi, in the former Soviet Union. *Macaca mulatta* monkeys were orally vaccinated with precipitated Boiven extract tablets of *S. flexneri* serotypes 2a, 3a, 4a, 4b, 6 and *S. sonnei*, and challenged three weeks later with a virulent *S. flexneri* 2a strain using 7.5×10^{10} CFU dose. Only 2 out of the twenty infected animals became sick while all the six of the control animals developed dysentery [26].

In these years it seemed to be imperative to establish a group focusing on the chemistry of the shigella O antigens. The primary goal was to establish the chemical structures of the *S. sonnei* phase I and II antigens. At the beginning of the 1960s – based on the work of Goebel – it was assumed that it contained only known sugars, particularly glucose, galactose and glucoseamine. The well-known phenomenon of phase-change was considered to be an S-R mutation, but it was not known whether or not the antigenic epitopes of phases I and II are carried by the same molecule. Using various extraction procedures we found that the determinants of the two phases are located on the same molecule [27, 28, 29]. During these studies we could detect further carbohydrate components, like L-glycero-manno-heptose and 2-keto-3-deoxyoctulonic acid (KDO) [30].

It was also shown that the lipid A-core-O-specific side-chain structure applies to *S. sonnei*, as well, the II phase determinants being the core structure, analogously to the *E. coli* R1 core. We found that the O-specific side chain is linked to the core by its C3 on its glucose component [31].

Working together with the coworkers of the Max Planck Institute of Immunobiology the structure of the lipid A of *S. sonnei* and that of the serologically similar *Plesiomonas shigelloides* was determined. Previously it was also shown that the sugar composition of the LPS of these two bacteria is very similar [32, 33, 34].

It was a considerable achievement to isolate a complete set of R derivatives from a phase II *S. sonnei* strain – a similar set so far available from *Salmonella* Minnesota, only. Furthermore, the set even included some "super R" mutants, too, containing only KDO-heptose or KDO only [35, 36–39].

Since the chemical studies required quantitative serological analysis a sensitive method called passive haemolysis inhibition was developed by simplifying the complement fixation reaction [40].

A member of the above-mentioned "R-set" of strains carrying only KDO-heptose contained D-glycero-D-manno-heptose instead of the L-glycero-D-manno-heptose. During the biosynthesis this is the precursor molecule of the latter one and its presence suggests the lack of a heptose-isomerase. From this and also from another mutant we could isolate ADP-D-glycero-D-manno-heptose and ADP-L-glycero-D-manno-heptose. These heptose-nucleotides are the substrates of the heptose-isomerase and -transferase and are the only nucleotide-activated carbohydrates [41, 42]. To separate them appropriate preparative and analytical chromatographic techniques were developed [43].

The O-specific side-chain expressing the phase I antigenic epitope could be separated from the whole LPS molecule and could be isolated in pure form by gel chromatography [31, 36, 44, 45]. An alternative technique was the use of ultracentrifugation of the phenol-water extract followed by ion-exchange chromatography. Using this latter technique we found that the O-specific side-chain – beyond the known sugar components present – contains other substances. Of these we could isolate a 2-amino-2-deoxy-L-altruronic acid. This was the first description of the natural occurrence of this molecule [46]. During this project an analytical gas chromatographic procedure was also developed to separate the eight possible aminoaldohexoses [47].

Parallel to these investigations genetic studies of *S. flexneri* were also part of the scientific panel of the Department. A K-12 F plasmid was successfully introduced into a *S. flexneri* 4b strain converting it to F+ [48]. Since shigellae do not express capsular material the antigenicity of the sex pilus could easily be studied and compared to similar pili coded by other plasmids [49]. Early attempts to isolate an Hfr derivative were unsuccessful. These led us to the conclusion – after Arber's work – that shigellae carry an *hsp* (today *hsd*) gene different from that of *E. coli* K-12 [50]. The restriction endonuclease coded by this gene digests the foreign DNA, as the T5 phage, F plasmid, or mobilized genomic DNA. We could transfer the *S. flexneri* *hsd* gene into an F+ *E. coli* strain. The conjugation of the so modified F plasmid into a *S. flexneri* 4b strain finally allowed the isolation of Hfr derivatives, and the determination of its integration site and that of the direction of its transfer. The genes localized by conjugation in *S. flexneri* proved to be allelic to the respective ones in *E. coli* K-12 [51]. These results

prompted us to investigate whether shigella strains are homologues in this respect. Although isolates could be divided in two groups, irrespective of the serotype, this has little relevance in taxonomy since *hsd* genes are known to be carried by phages, too [52].

It was described that the basic structure of the O-specific side chain of *S. flexneri* is the so-called Y variant coded by the *rfb* gene on the chromosome, while the serotypes are the results of lysogenic conversions. From a serotype 4b strain we isolated a converting phage (IV1 antigen) and compared it to Iseki's IV phage [53]. These experiments initiated our comparative studies with various converting phages. It was shown that these phages have similar electron microscopic morphology (Bradley group A) and are closely related to each other as was shown by cross-neutralization studies [54]. We found that lysogenic conversion leading to the modification of the O-specific side chain enhances virulence by increasing the intraepithelial growth capacity [55]. Using thermo-sensitive mutants of the converting phages the mechanism of conversion was revealed, i.e. the phage genome codes for a α -glucosyl transferase and activates polyprenyl phosphate glucose synthetase [56]. It should be noted that these experiments confirmed the antigenic scheme proposed by Rauss differentiating type antigens (derived from lysogenic conversion) from group antigens. This project also resulted in the isolation of a converting phage coding for the type V antigen (PE5). Its generalized transducing capacity was shown, as well as its integration into the proline region of the chromosome leading to specialized transduction [57].

The enterotoxins were described in the 1970s. We could detect a heat-stable enterotoxigenic activity in *S. flexneri* [58]. It was shown that its apparent high molecular weight is not due to complex with the LPS molecule. It was immunogenic and its effect could be neutralized with anti-LT antibodies [59]. Beyond its enterotoxigenic activity its cytotoxic effect was also demonstrated [60].

The fact that infection of the epithelial cells of the large intestine is a central step in the pathogenesis of bacillary dysentery has been known since the 1960s [61]. However, it was in the early 1980s only that the association of its genetic basis with the carriage of a large extrachromosomal element, called the invasion plasmid (pINV) was demonstrated. The presence of the pINV was shown in all invasive strains of the four species of the *Shigella* genus, as well as in the enteroinvasive *E. coli* (EIEC) strains [62, 63, 64]. Being interested in the virulence related antigens of shigella and EIEC our assumption was that if all these bacteria cause the same diseases (i.e. bacillary dysentery) by the same mechanism (i.e. by infecting colonic epithelial cells) and harbor the same virulence related genes (i.e. the pINV) they may also express similar or identical antigen(s). If doing so, prospectively, an antigenic moiety of this kind could

be utilized as target for immunoassays, and could serve as antigenic candidates in future vaccines.

Indeed, using an absorbed polyclonal rabbit immune serum the expression of a species- and serogroup-independent, virulence specific antigen was proven and was tentatively called virulence marker antigen or VMA [65]. It was shown to be coded by the invasion plasmid [66]. As more data became available on the genes present, and on the proteins coded for by the pINV, the VMA was demonstrated to be identical with invasion plasmid coded antigen C (IpaC) [67].

Attempts were made to utilize the VMA specific antibodies for the recognition of pathogens causing dysentery. Early studies showed that a simple VMA specific ELISA test specifically differentiated EIEC strains isolated from children from non-EIEC isolates [68, 69]. To circumvent the technical difficulties associated with the production of the polyclonal antibodies a monoclonal antibody against the IpaC antigen (MAIC-1) was produced while one of us (T.P.) was working at the Department of Clinical Microbiology, Karolinska Institute, Stockholm [70]. An ELISA using this antibody was used successfully to detect shigella and EIEC strains in clinical samples [71] and to recognize invasive *E. coli* belonging to serogroups previously not identified as EIEC [72]. The technique was also converted into a colony blot assay making it suitable to detect these groups of pathogens from liquid samples, like water, with high specificity and sensitivity [73].

In order to prove that the invasion plasmid coded antigens are expressed *in vivo*, too, early attempts were made to detect serum antibody response against them in dysenteric patients. Using an ELISA technique it was shown that patients infected with *S. sonnei* do develop high titer serum IgG response against this set of antigens [74]. Later it was also demonstrated that serum and colostrum anti-Ipa antibody levels correlate with previous exposures to bacillary dysentery infections making the method suitable for sero-epidemiological studies [75, 76]. The usefulness of the technique for the evaluation of the immunogenicity of live attenuated anti-dysentery vaccines was also demonstrated [77].

The *in vivo* expression of the invasion-related Ipa proteins raises the possibility that they could be part of a future anti-dysentery vaccine. Plasmid coded cell-components promote the initial interaction of bacteria with target cells [78], as well as the capacity of the pathogen to move intracellularly [79]. The presence of antigens shared by all dysentery-causing bacteria carries the promise to elicit protection against a broader scale of pathogens than the serotype specific LPS-based vaccines. Anti-Ipa antibodies indeed were shown to prevent cell-invasion by shigella or EIEC, at least *in vitro* [80], and animal experiments have also provided some evidences that this goal may eventually be attainable [81].

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A SHORT HISTORY OF MICROBIOLOGY IN HUNGARY FROM THE BEGINNING TO 1951*

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Introduction

“On December 27th 1927 at a committee meeting of the Royal Society of Natural Sciences, **Dr. Hugó Preisz M.D.** proposed the foundation of a microbiological section within the Society. He commented that the previous year at an international conference on rabies in Paris, several scientists decided to establish an international microbiological society with the aim of promoting scientific work in each branch of microbiology and fostering friendly relations between scientists working in different countries. The Hungarian microbiologists called together regarding this matter founded the **Hungarian Society for Microbiology**. They elected retired university Professor **Hugó Preisz M.D.** president...”

Hugó Preisz M.D. asked whether it would be more reasonable to establish a **Microbiological Section**, within the framework of the **Royal Society of Natural Sciences**, instead of an independent Hungarian Society for Microbiology. The committee accepted the suggestion unanimously and elected **Aladár Aujeszky M.D.** vice-president, **Lajos Gózon M.D.** secretary and **Béla Johan, M.D.** and **Rezső Manninger D.V.M.** editors.

We can learn about the activities of the Microbiological Section of the Society of Natural Sciences from the short reports appeared in the issues of the Bulletin of the Royal Society of Natural Sciences. We know from these short reports that in 1927 seven Hungarian microbiologists participated in a microbiological conference held in

* This paper was written to commemorate to the fiftieth anniversary of the foundation of the Hungarian Society for Microbiology.

Vienna. These scientists were: **László Detre, Béla Fenyvessy, Béla Kanyó, János Köves, Miklós Rohonyi, Lajos Szélyes and Károly Vas.**

The following years did not serve to strengthen the newly formed Section. **Aujeszky** died in 1933 and **Preis** in 1940. Some fled abroad from anti-Jewish discrimination, others chose inland seclusion within the country. After the Second World War the class struggle, nationalisation and the complete transformation of our scientific life justified the foundation of a new, independent **Hungarian Society for Microbiology**. The 50th anniversary would not be complete without mentioning the achievements in microbiological research of outstanding Hungarian scientists in the decades before the foundation on one hand and a re-examination of the foundation year of the Society in relation to its **legal continuity**.

Institutes of microbiology and famous microbiologists in the decades preceding the establishment of the Society

Institutes

From the point of view of the development of microbiology in Hungary, the laboratories and institutes, ensuring the provision of the equipment necessary for the cultivation of this special field, are of crucial importance. The most important of them are the many **vaccine producing** institutes, the first of which, the *Smallpox Vaccinations Institute*, was opened in 1824 for the purpose of producing vaccine against **pox** under the direction of Professor **Ferenc Xavér Gebhardt**. The *Pasteur Institute* of Budapest, established at the suggestion of Professor **Endre Hőgyes** and run by him until his death, is of immense significance from the point of view of both the development of microbiology and the history of national vaccine production. The institute began its activity on April 15, 1890 following order No. 57404 of January 18, 1890 issued by the Ministry of Religion and Public Education.

In the same year when **Hőgyes** started the use of vaccine against rabies (1890), **István Bethlen**, the minister of agriculture, signed an agreement with the director of the Vienna branch of the *Pasteur-Chamberland* laboratory, as a result of which the laboratory of dubious fame was transferred to Budapest. Control of the vaccine against *anthrax* and *swine erysipelas* distributed by the laboratory in our own country was carried out by Professor **István Rátz**, the director of the Institute of Veterinary Pathological Anatomy. The start of home production of veterinary vaccines is linked to the name of Professor **Preis** and is connected with the activity of the *Royal Hungarian Institute for Bacteriology* founded by him in 1890.

As it can be seen from what has been said so far, following the birth of bacteriology, several laboratories of the new science were opened throughout the country; **Kálmán Balogh's** *Institute for General Pathology*, **Gustav Scheuthauer's** *Institute for Pathological Anatomy*, **Fodor's** *Institute for Public Health within the University* and the *Metropolitan Institute for Bacteriology*. The latter carried out considerable researches and investigations under the direction of **Ottó Pertik**. The very first institute with national authority and influence was the *Royal (State) Hungarian Institute for Bacteriology* mentioned above, which was firstly led by **Hugo Preisz** and later by **Aladár Aujeszky**. This institute performed the official diagnostic and research tasks relating to microbiological examination until the *National Institute for Public Health* was opened under the direction of **Béla Johan** in 1927. Following this model the *National Institute for Veterinary Hygiene* began its activity in 1929 under **Rudolph Manninger's** guidance. More and more serum and vaccine producing institutes were established. University clinics, pathological institutes and hospital pathology departments opened microbiological laboratories. Besides, specialists required specific laboratory qualifications and needed to acquire the necessary microbiological knowledge to become laboratory specialists.

Despite the widespread, successful microbiological activity a long time passed before a department of microbiology was established within a university. It first came into existence at the then Veterinary High School (1907) under the direction of **Aladár Aujeszky**. At the Medical Faculty of the University of Budapest the *Institute for General Pathology and Bacteriology* was founded in 1883 under the direction of Professors **Endre Hőgyes**, **Hugó Preisz** and later **Sándor Belák**.

After the end of the Second World War, independent medical universities were established with independent microbiological institutes. At the Medical University of Budapest, the name of the former Institute for General Pathology and Bacteriology was renamed the *Institute for Bacteriology and Immunology* and the gifted **Ferenc Faragó** was appointed its director. After his tragic death in 1950 the *Institute for Microbiology* was established under the direction of Professor **Zoltán Alföldy** (1904–1992) and the institute has been successfully serving medical training needs ever since.

As in the case of the Medical University in Budapest, the Medical Universities of Pécs, Szeged and Debrecen similarly founded a Department of Microbiology under the direction of Professors **Béla Fenyvessy**, **Károly Rauss** (Pécs), **György Ivánovics** (Szeged), **Endre Jeney** and **Lajos Váczi** (Debrecen), respectively. Significant microbiological activity was characteristic of the Institutes of Hygiene of the Medical Universities. Their activity, however, belongs to the 2nd phase of the development of microbiology in Hungary.

Bacteriologists

Having discussed the birth and development of these microbiological institutions we consider it important to review the names and activities of those Hungarian scientists and researchers who contributed creatively to the universal development of this branch of science and who can rightly be called the Hungarian masters of microbiology. The significance of their activity is increased by the fact that their appearance demanded a new approach not only to medicine but to their way of thinking, which was not received favourably by all members of the scientific community.

They had to continue a difficult struggle with the then fashionable alternative trends. Among these in particular were the followers of homoeopathy, who denied the aetiology of illness and disagreed with the ideas of infection and its role, offering medicine for every illness on the basis of their peculiar system of fallacious notions. The spread of their false doctrines was aided by the cholera situation, against which academic medicine was still helpless. Having the new results of bacteriology accepted in such a situation demanded the energy and charismatic personality of **Kálmán Balogh**, Professor of Pathology (1835–1888). It was he who under his microscope first observed bacteria in the blood smear of a sheep perished by anthrax (1863). Not only did he become the first enthusiastic follower of bacteriology and the theory of infection, but he also encouraged his colleagues to do microbiological research.

Remarkable bacteriological and parasitological investigations were begun first in **Gusztáv Scheuthauer's** (1832–1894) Institute of Pathology and later in **József Fodor's** (1843–1901) Institute of Public Health. This demonstrates the two main trends in the development of microbiology, the **pathological** and the **hygienic** directions. The former studied the pathogenic characteristics of microbes from the point of view of **pathogenesis** of diseases. The latter dealt partly with **diagnostic** and partly **with serum and vaccine producing** tasks in order to prevent **epidemics**.

Joseph FODOR (1843–1901) was the founder of public health research in Hungary, and became the first Professor of hygiene as the pioneer of bacteriological tendency in hygiene. In the 1870s he found that soil is the most abundant in bacteria in its upper layer, and that the decomposition processes in the soil are closely related to bacterial density. During the typhoid epidemic affecting the town of Pécs in 1890, he found the source of infection in the sewage oozing from the privy of the hospital laundry into the trunk line of the water supply. Studying the bacterium-filtering ability of the soil, he searched for possibilities of accidental bacterial contamination of dug wells. He constructed equipment suitable for lifting out samples from different depths for bacteriological examination, and another apparatus, the so-called "bacterium-fishing" one (later regarded as an ancestor of the micromanipulator) for the isolation of

bacterial colonies and production of pure cultures. He showed in 1886 that, due to the bactericidal action of blood, bacteria injected into the blood of experimental animals were quickly decomposed there. For this last discovery he is regarded as the first immunologist, the discoverer of humoral immunity.

Victor BABES (1854–1926) Rumanian microbiologist, founder of Rumanian microbiology, began his professional career in Scheuthauer's Institute of Pathological Anatomy. He wrote a number of publications in Hungarian, for example the first textbook of *Bacteriology* (1886). Rumanian authorities, taking note of his valuable research work (i.e. on *Corynebacteria*) performed in Budapest, invited him to continue the microbiological research in Bucharest, Rumania. His successful achievements are of essential importance.

Hugó PREISZ (1860–1940) was the founder and first Director (1890) of The Royal State Institute of Bacteriology in Budapest, Hungary. He soon became the first Professor of General Pathology and Bacteriology at the Faculty of Medicine of Budapest University. He was interested in practical bacteriological diagnostics, vaccine production and control. He was the first to initiate research on theoretical questions of immunology. Using intradermal injections he studied virulence of swine erysipelas bacteria, a species that had been discovered by Loeffler two years earlier. The method developed by him became widely accepted on the basis of the publication of his co-workers **FORTNER** and **DINTER** (1944). He revealed that strains of the causative agent of swine erysipelas that had lost virulence due to *in vitro* passages regained virulence while passaged in pigeons. He discovered *Corynebacterium ovis* called *Bacillus Preisz-Nocard* for a long time. He also worked with the bacteria called *Yersinia* today. The experience at his plague laboratory helped to stop the panic that had broken out in Fiume because of plague-suspect cases observed in 1901. The discovery that virulence of *Bacillus anthracis* is associated with the capsule-producing capacity of the strains was of outstanding importance. His results on the S/R variation of *Bacilli* founded an international school of microbiology with the participation of **Joseph TOMCSIK** and **György IVANOVICS** in Hungary, and many other colleagues abroad. He also worked with *Brucella* strains. He developed the Preisz' pyrogalllic acid procedure for the cultivation of bacteria under anaerobic conditions. He contributed to the discovery of *Brucella suis* by **Ferenc HUTYRA** and **Árpád MARCIS** differentiating it from *Brucella abortus*, to the recognition of the human pathogenicity of *Mycobacterium bovis* recognizing the bacterium contamination of commercial milk products. His work on bacteriophages will be mentioned below.

Aladár AUJESZKY (1869–1933) was the successor of **Preisz** as Director of the Royal State Institute of Bacteriology. He was elected Professor of Bacteriology of the Veterinary High School of Budapest. He had innovative results on tuberculosis,

malleus, fowl typhoid, anthrax and staphylococcus bacteria. His most important discovery was in the field of virology, which will be mentioned later.

László DETRE (1874–1939) invented the word “antigen”. He was Director of the Jenner-Pasteur Laboratory (1901–1918) and of the “Hungarian Serum Works”. He became senior lecturer at the University later. He discovered the bacterium now called *Clostridium perfringens* variant C. He succeeded in differentiating the infection of this bacterium called by him “*Bacillus zoodysentariae* (Hungaricus)” from the clinical picture of coli dysentery.

Gyula DARÁNYI (1888–1958) was the Professor of Hygiene at the Faculty of Medicine of Budapest University. He worked with *Staphylococci*, *Mycobacteria*, and on the thermoresistance of bacteria. He wrote an outstanding handbook of hygiene including the 3rd volume on bacteriology.

Rezső MANNINGER (1890–1970). Professor of Epidemiology and Microbiology at the University of Veterinary Science, Budapest. He was an important member of the Hungarian school of microbiology research. He discovered many properties of the virulence of *Pasteurella* strains, contributed to the development of swine erysipelas vaccine strains, and to the typing and taxonomy of *Salmonella* isolates, he transformed successfully *Bacillus* strains with the DNA of capsule-producing strains of *Bacillus anthracis* in 1962.

István LOVREKOVICH (1895–1960) was the main organizer of the department of Bacteriology at the National Institute of Public Health, Budapest in 1927 financed by The Rockefeller Foundation. He developed a bismuth-sulphite-agar for the differential diagnostics of *Salmonella typhi*. Using this medium, he raised typhoid diagnostics to an outstandingly high level. In cooperation with **Károly RAUSS** he developed a typhoid vaccine of high efficacy. After World War II he worked in a Hungarian pharmaceutical factory. He initiated Hungarian streptomycin production, and performed successful investigations into other antibiotics.

Ferenc FARAGÓ (1905–1950). Professor and director of the first chair and Institute for Bacteriology and Immunology – in collaboration with his master Joseph TOMCSIK (1898–1964) – achieved considerable success by vaccination against diphtheria. The “depot” method raised satisfactory immunity in a single inoculum. He produced also considerable results in the field of immunization against pertussis and scarlet fever. He died in 1950 as a victim of a political made-up charge.

Zoltán ALFÖLDY (1904–1992). Professor of Microbiology of the Semmelweis University School of Medicine, Budapest, initiated the research on *Leptospira* in Hungary in 1947 with the group led by him. It has to be mentioned that thousands of Hungarian medical students were taught using the Textbook of Microbiology written by him with his co-authors.

Rickettsia researchers

Rickettsia research in Hungary served the purpose of vaccine production against exanthematous typhus. In the beginning body lice were used for the propagation of pathogens. The parasitologist **Ferdinand Ziegler** (Nándor Zoltai) excelled in this work. Later **Elek Farkas** and his co-workers succeeded in adapting the Matelska-strains brought from Berlin to embryonated hen's eggs. Due to the administration of the Cox-type vaccine produced in this way, our country remained free from the exanthematous typhus, a frequent concomitant of war.

Food microbiologists

In the field of bacteriological meat-inspection **Albert Breuer** (1870–1930) and **Géza Semsey** (1894–1947) and in the field of microbilological examination of milk **Ottó Fettick** gained the greatest distinction.

Plant microbiologists

The activity of the high school chemistry teacher **Mór Preys** (1829–1877) is of significance, not only from the point of view of the history of Hungarian microbiology, but also from that of universal microbiology. In 1861 at almost the same time as Pasteur, but independently, he described the possible conservation of wine by heating.

Mention must be made of the great achievements such as

Gyula Istvánffy showed great merit in the investigation of the diseases of grape vines.

Károly Schilberszky (1863–1933), **Károly Vas** (1884–1948) and **László Beke** (1881–1950) were prominent in the study and explanation of the aetiology of the potato and other plant diseases.

Dániel Fehér (1891–1955) distinguished himself in the field of soil bacteriology.

Virologists

In the struggle against diseases of viral origin, **János BÓKAY JR.**, professor of pediatrics (1858–1937) distinguished himself at a very early stage. In 1892 he observed the similarities, moreover identity of varicella (chickenpox) and herpes zoster (shingles).

Endre HÓGYES (1847–1906), Professor of General Pathology at Budapest University, established the Institute Pasteur of Budapest and he was the first Director of that institute. He developed and characterized a new, original fixed virus strain of rabies virus and found the strain highly suitable for vaccine production. Furthermore, he introduced a so-called "dilution" technique, which proved to be preferable to Pasteur's method based on exsiccation of spinal cords. His successful investigations were supported by his inventive and ambitious co-worker, **József LÓTE** (1856–1938).

Aladár AUJESZKY (see above) was another successful pupil of **Endre HÖGYES**. He discovered a form of encephalitis now called "Aujeszky disease" (he used the designation "pseudorabies") (1902). He isolated the virus of the disease and described some of its properties. These investigations added considerable data to the differential characterisation of the fixed virus of rabies and its differentiation from the street virus.

The swine plague ravaging in Hungary at that time prompted **Ferenc HUTYRA** (1860–1934), Professor of Epidemiology at the Veterinary High School, to establish a virus research laboratory in which he and his co-worker, **János KÖVES** (1882–1977) isolated the pathogen of the disease. They characterized the virus biologically and developed an immunisation method, which was applied successfully to the control of swine plague for many years. Their so-called "simultaneous" method of immunisation was taken over by a number of countries.

It was in 1907 that **József MAREK** (1868–1952), Professor of Internal Medicine of the Veterinary High School, Budapest, discovered the viral neuromyelomatosis of hens, now called "**Marek's disease**"; he infected hens with the virus successfully.

As to the investigations into the cytopathogenicity of viruses, **György IVANOVICS** (see above) and the American, **HYDE** used micromorphological changes of cells as indicators of *in vitro* virus infection at a very early time (1936); **Rezső MANNINGER** and **Ferenc LÁSZLÓ** (1930) reported on genetic changes in the foot-and-mouth disease virus while **Rezső MANNINGER**, **József CSONTOS** and **Gyula SÁLYI** (1939) isolated the suipox virus and proved its independence. **Rezső MANNINGER** and **József CSONTOS** (1940) proved the identity of the virus of catharrhal influenza of horses with that of "viral abortion". **György IVÁNOVICS** and his co-workers reported on the cyclic reproduction of the Aujeszky virus.

Among the other masters of virology, **Károly JÁRMAI** (1887–1941) and **Gyula TAKÁTSY** (1914–1980) deserve special attention; both devoted their whole scientific activity to virus research.

Károly JÁRMAI, Professor of Pathological Anatomy at the Veterinary High School, Budapest, published considerable results on the viruses of bovine papillomatosis, fowl sarcoma and other transferable tumour viruses. His investigations into fowl leukoses were of special importance. His research fundamentally contributed to the differentiation and biological characterization of the viruses causing lymphogenous and myelogenous leukosis as well as erythroleukosis of the fowl.

To make **Gyula TAKÁTSY's** merits easier to understand, let us outline the history of influenza research in Hungary from virological aspects.

After the early attempts of **Lajos FEJES** (1919) to infect monkeys with the hypothetic virus, the pathologist **E. BALOGH** proclaimed the viral aetiology of influenza on the basis of his own research (1922–1942). In the mid-thirties, the Rockefeller Foundation established an influenza research laboratory with the American **Richard M. TAYLOR** (1887–1981) as leader, in the National Institute of Hygiene, Budapest. Soon the first influenza virus strain from Central Europe was isolated (**R. Taylor** and **M. Dreguss**, 1936); the susceptibility of *Cricetus cricetus* was proved (**Taylor** and **Dreguss**, 1940) and the antigenic effect of the virus was measured (**Dreguss**, 1940) in the same laboratory. **M. Dreguss** and **Ferenc Hoffmann** (1941) succeeded in immunizing domestic pigs, then **M. Dreguss**, **Árpád Hegyeli** and **József Szathmáry** (1943) reported on natural infection of pigs with the human influenza virus.

The influenza virus research was strikingly promoted, at first in Hungary, then all over the world, by the use of the Microtitrator Set, which was developed by **Gyula TAKÁTSY** in 1949 and published in the early 1950s (**J. Takátsy**, 1950, 1952, 1955). The set has been advantageously applied not only in virology but also in microtechniques necessary in many other branches of science; both in research and practice. In the National Institute of Hygiene, researchers at the Department of Virology soon isolated and characterized influenza virus strains of different serotypes. A fruitful collaboration was built up with the World Influenza Centre, London. **Julius Takátsy** studied the role of antigen variants in epidemics thoroughly and developed a simple new method for purification and concentration of influenza virus (1954) and applied his micromethods in developing a serum-absorption test aimed at analysing the antigens of different strains with high accuracy. He extended his research to the ecology of influenza virus and developed a method suitable for mass production of influenza vaccine even in poor countries; he followed the efficiency of mass vaccination using his simple methods.

Phage researchers

At the top of the list of Hungarian phage researchers the name of **Hugó PREISZ** is to be found again (see above). He initiated investigations soon after d'Herelle's discovery (1917) and supported the virus theory of phages definitely. In reports dealing with the so-called *Pettenkoferia* he distinguished the microorganisms from phages. He summarized his phage research in a monograph. Phage typing of bacterial strains started in the National Institute of Hygiene in a laboratory headed by **Mária EÖRSI** (1948).

An investigator of Chlamydia

Miklós MELCZER (1891–1985), Professor of Dermatology and Venereal Diseases first at the Faculty of Medicine the University of Szeged, later Pécs,

investigated the aetiology of lymphogranuloma venereum, applying Loeffler's method and later, successfully, the Herzberg method. He reported on intracellular cumulative location of the pathogen. These investigations were performed as early as 1934, i.e. before **Tamura** (1935) and **Miyagawa** (1935) reported on the discovery of the pathogen. For this reason, Melczer's results were called into doubt, even in his home country, and the pathogen was given the name *Miyagawanella* (present name: *Chlamydia*). In a monograph (1983), Melczer summarized the biological – including microscopical and cultural – characteristics of the pathogen, its animal pathogenicity, etc.

Mycologists

On the top of the world list of medical mycologists two investigators of Hungarian origin are to be found. These are **Dávid GRUBY** (1810–1898) and **Lajos MANDL** (1812–1881). Both lived in Paris.

Dávid GRUBY together with **Lajos Mandl** as helpmate constructed a microphotographic equipment to his microscope, thus he can be regarded as the inventor of microphotography, which was also popularized by him. Among pathogenic fungi, *Achorion*, *Trichophyton*, *Microsporon*, *Oidium* and *Streptothrix* were discovered and described by **Dávid Gruby**. Moreover, he described the clinical pictures caused by fungi, created nosological groups and gave them definitive names, thus stressing their specificity.

Gruby's epoch-making discoveries became widely known only after **Sabouraud's** publications. In **Lajos NÉKÁM's** dermatological clinic, wide-range mycological activities were in course with the leadership of **Ede NEUBER** and later **BALLAGI**. In an excellent monograph written by **BALLAGI** (1929), the whole scope of contemporary medical mycology is included.

With endogenous (systemic) mycoses mainly pathologists were dealt with initially, first of all from diagnostic, pathogenetic, pathologic and pathohistological aspects. Mycology got a new impulse in Hungary when, soon after the end of World War II **Béla JOHAN** (1889–1983) was charged with initiation of penicillin production. Not much later (1955), a mycological laboratory was established in the National Institute of Hygiene. Then, after the importance of mycotoxicoses had been recognized, research was initiated in order to clarify the corresponding clinical pictures.

Protozoologists

In the list of Hungarian protozoologists, **Dávid GRUBY** was also the first; he discovered the protozoon parasite *Trypanosoma sanguinis* (1842). **Géza ENTZ** (1842–1919) presented another discovery of great importance: he was the first to report that protozoa and algae were living together (1875). It had long been known that some protozoa contained formations that were generally thought to be chlorophyll granules. **ENTZ** discovered that the granules were, in fact, algae. Later, this sort of co-partnership was designated as symbiosis by the German investigator **BRANDT** (1981). By his discovery, **Entz** elucidated the way towards recognition of **intracellular parasitism**.

The Rumanian microbiologist, **Victor BABES**, at that time Professor in Hungary (see above) made a rather notable discovery in protozoology.. He was requested by the Romanian authorities to study the epizootic haemoglobinuria of sheep grazing in territories exposed to swellings of the Danube, where 20% of the sheep had died of disease. **Babes** (1888) found parasitic protozoa in the erythrocytes of sick sheep. Later, he elucidated its intracellular parasitism, and pathogenicity. His priority was recognized by the designation of the parasite: family of *Babesia*.

István RÁTZ (1860–1917), Professor of Pathological Anatomy at the Veterinary High School, Budapest, was the promoter of parasitology in Hungary and its outstanding cultivator even in international relation. His *Sarcosporidium* studies deserve special interest; in his reports, two parasitic protozoa are described and their taxonomic place is presented.

Miklós JANCsó (1868–1950), Professor of Internal Medicine at the Faculty of Medicine of the Ferenc József University (Szeged), became famous mainly because of his malaria research. He studied among other things the development of malaria plasmoids in the mosquito and determined the effect of temperature on different developmental stages.

Ferenc LŐRINCZ (1898–1986), the organiser and first leader of the Department of Parasitology of the National Institute of Hygiene also owed his reputation first of all to malaria research. He was very active in the eradication programme.

Sándor KOTLÁN (1887–1967), Professor of Parasitology at the Veterinary High School, a world-famous parasitologist, deserved the title “master of Hungarian parasitology” by the discovery of numerous protozoon species. He described previously unknown *Eimeria* species living in sheep, rabbits and geese; he clarified the course of endogenic development of numerous *Eimeria* species with special regard to globidium schizogeny. He resolved some aetiological and vector problems of pyroplasmosis. The

Coccidium research initiated by KOTLAN was continued by László PELLÉRDY (1907–1974).

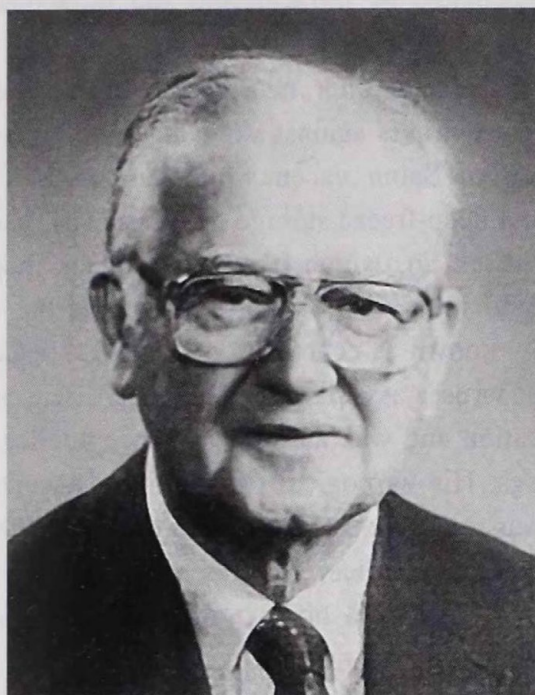
Hungarian microbiologists had to face the difficulties presented by two world wars. Hungarian microbiologists still had a significant effect in the fields of research and education at home and abroad. As a result of their hard work the independent Hungarian Society for Microbiology was founded in 1951 under the presidency of András Havas.

Selected original handbooks, textbooks and practical manuals of microbiology from Hungarian authors in this period

1. Babes, V.: A bakteriológia rövid tankönyve. (Short textbook of bacteriology) Magyar Orvosi Könyvkiadó Társulat, Budapest, 1886.
2. Huttya, F.: A háziállatok fertőző betegségeinek oktana. (Infectious diseases of domestic animals) Atheneum, Budapest, 1888.
3. Tangl, F.: Útmutató a bakteriológiában. (Guidebook of bacteriology) Dobrowsky és Franke, Budapest, 1894.
4. Tangl, F.: Bakteriológia. (Bacteriology) In: Tausz, F. (ed.): Klinikai diagnosztika (Clinical diagnostics) II. pp. 469-544. Dobrowsky és Franke, Budapest, 1894.
5. Preisz, H.: Bakteriológia. (Bacteriology) Magyar Országos Állatorvos Egyesület, Budapest, 1899.
6. Pertik, O.: Bevezetés a fertőző betegségek kóroktanába bakteriológiai szempontból. (Pathogenesis of bacterial diseases) In: Bókai- Kétli- Korányi: A belgyógyászat kézikönyve (Manual of internal medicine) I. pp. 1- 98. Magyar Orvosi Könyvkiadó Társulat, Budapest, 1894.
7. Udránszky, L.: A baktériumok kémiai terményeinek szerepe a fertőző betegségeknél. (Bacterial products in infectious diseases) In: Bókai- Kétli- Korányi: A Belgyógyászat Kézikönyve (Manual of Internal Medicine) I. pp. 95- 136. Magyar Orvosi Könyvkiadó Társulat, Budapest, 1894.
8. Högyes, A.: Lyssa. (Rabies) In: Nothnagel (ed.): Spec. Pathol. u. Therapie, Bd. V. Teil V. Zoonosen (Zoonoses) II. Abth., Hölder, Wien, 1897.
9. Kaiser, K.: A bakteriológia rövid tankönyve. (Short textbook of bacteriology) Dobrowsky és Franke, Budapest, 1899.
10. Rigler, G.: Közegészségtan és a fertőző betegségek (Hygiene and infectious diseases) II. kötet. Kolozsvár, 1910.
11. Aujeszky, A.: A baktériumok természetrajza. (Natural history of bacteria) Királyi Magyar Természettudományi Társulat, Budapest, 1912.
12. Aujeszky, A.: Általános bakteriológia. (General bacteriology) Kir. M. Természettud. Társ. Budapest, 1924.
13. Gózon, L., Lénárd, V.: A gyakorlati bakteriológia zsebkönyve. (Pocket handbook of practical bacteriology) Érseki Lyc., Eger, 1913.
14. Ballagi, I.: Bőrgyógyászati mykológia. (Dermatomycoses) Magyar Orvosi Könyvkiadó Társulat, Budapest, 1920.

15. Baló, J.: A láthatatlan kórokozók, filtrálható vírusok. (Invisible agents, filtrable viruses) Magyar Orvosi Könyvkiadó Társulat, Budapest, 1931.
16. Lovrekovich, I., Tocsik, J., Lőrincz, F.: Bakteriológia, immunitástan és parazitológia. (Bacteriology, immunology and parasitology) Magyar Orvosi Könyvkiadó Társulat, Budapest, 1935.
17. Melczer, M.: A negyedik nemi betegség. (The fourth sexual disease) Magyar Orvosi Könyvkiadó Társulat, Budapest, 1938.
18. Lőrincz, F.: A maláriáról. (Malaria) Magyar Orvosi Könyvkiadó Társulat, Budapest, 1939.
19. Darányi, Gy.: Közegészségtan (Hygiene) III. Magyar Orvosi Könyvkiadó Társulat, Budapest, 1940.
20. Láng, S.: Bakteriológiai és serológiai vizsgálatok. (Bacteriological and serological tests) Novák R. Budapest, 1941.
21. Kotlán, S.: Parazitológia. (Parasitology) Magyar Országos Állatorvos Egyesület, Budapest, 1944.
22. Faragó, F.: Bakteriológia és immunitástan. (Bacteriology and immunology) Magyar Orvosi Könyvkiadó Társulat, Budapest, 1948.

IN MEMORIAM JOSEPH L. MELNICK
(1914–2001)



Professor Joseph L. Melnick, 86, Dean Emeritus of the Graduate School of Biomedical Sciences and Distinguished Service Professor of virology and epidemiology at Baylor College of Medicine, Houston died on 7 January 2001. "It was my good fortune to have been a scientist during the golden age of virology, when new techniques were being introduced into the field." He wrote this in an article some years ago. The reality is, however, that he had been one of those highly gifted scientists whose activities actually resulted in golden age of virology from the early 1950s of the last century.

He was a Ph.D. graduate of Yale University and worked there between 1942 and 1957, when he became the chief of Virus Laboratories in NIH, Washington D.C. and then in 1958 joined the Baylor College of Medicine as professor and founding chairman of the Department of Virology and Epidemiology. Under his leadership that Department merited international reputation for research and education in virology and thus became the WHO Collaborating Center for Virus Reference and Research.

He was a pioneer polio researcher. He was among the first to demonstrate that poliovirus usually invades the human intestinal tract rather than the central nervous system and that majority of infections remain clinically silent. He intensively participated in studies connected with live poliovirus vaccines including among others comparison of the Sabin strains and the Lederle-Cox strains for neurovirulence in hundreds of monkeys inoculated by intracerebral and intraspinal routes, the genetic stability of attenuated polioviruses after multiplication in man, the correlation of *in vitro* markers of polioviruses and their neurovirulence for monkeys and men, the interference caused by enteroviruses against attenuated polioviruses. He elaborated the $MgCl_2$ thermostabilization of Sabin vaccine making possible the immunization of people in countries without deep-freeze storage facilities. The discovery of members of the large group of viruses to which the polioviruses belong bound him to characterization and classification of enteroviruses such as the viruses first called as "orphan viruses" and now known as echoviruses. His work began with polio research but spanned wide scale of viruses associated with human diseases. He had a permanent interest in virus classification and was the first to conceptualize, classify and name a number of virus groupings. His virological publications exceeded 1000. His Medical Microbiology textbook was regularly updated and in its 22nd edition has been translated into many languages for use by medical students around the world. His dedication to training young scientists resulted in a number of recognized virologists all over the world including also Hungary.

Professor Melnick had been a member of WHO Expert Advisory Panel on Virus Diseases for more than 30 years and was active in WHO programs concerned with entero-, hepatitis- and herpes-viruses. He was regularly the chairman on annual meetings of WHO Consultative Group on Oral Poliomyelitis Vaccine established in 1972 to supervise the use of seed viruses of Sabin strains for vaccine production and to follow up the results obtained with the vaccines applied. On these meetings and on those held in WHO Headquarters between 1967 and 1970 he took a special interest in studies carried out in Hungary on safety and efficacy of Sabin vaccine applied in annually repeated nation-wide campaigns presented by the Hungarian member of the expert group. This played a certain role in his decision as the secretary-general to hold the 2nd International Congress of Virology in Budapest in 1971. That was the historical meeting when the Virology Section of International Association of Microbiological Societies has been established and Professor Melnick became the elected first chairman. He later on visited Hungary several times, last time in 1996, when he had been the chairman of a WHO meeting in Siófok connected with prevention of hepatitis B infections.

The Hungarian virologists will persistently keep him in mind as an international leader in the identification and prevention of viral diseases and one of creators of golden age of virology.

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